

## OSTEOPROTEGERIN

This application is a continuation of  
Serial No. 09/132,985 filed 8/12/98, which is a  
5 continuation of Serial No. 08/771,777, filed 12/20/96,  
which is a continuation in part of 08/706,945, filed  
9/03/96, which is a continuation in part of 08/577,788,  
filed 12/22/95 which are hereby incorporated by  
reference.

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Field of the Invention

The invention relates generally to  
polypeptides involved in the regulation of bone  
metabolism. More particularly, the invention relates  
15 to a novel polypeptide, termed osteoprotegerin, which  
is a member of the tumor necrosis factor receptor  
superfamily. The polypeptide is used to treat bone  
diseases characterized by increased bone loss such as  
osteoporosis.

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Background of the Invention

Polypeptide growth factors and cytokines are  
secreted factors which signal a wide variety of changes  
in cell growth, differentiation, and metabolism, by  
25 specifically binding to discrete, surface bound  
receptors. As a class of proteins, receptors vary in  
their structure and mode of signal transduction. They  
are characterized by having an extracellular domain  
that is involved in ligand binding, and cytoplasmic  
30 domain which transmits an appropriate intracellular  
signal. Receptor expression patterns ultimately  
determine which cells will respond to a given ligand,  
while the structure of a given receptor dictates the  
cellular response induced by ligand binding. Receptors  
35 have been shown to transmit intracellular signals via  
their cytoplasmic domains by activating protein

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tyrosine, or protein serine/threonine phosphorylation (e.g., platelet derived growth factor receptor (PDGFR) or transforming growth factor- $\beta$  receptor-I (TGF $\beta$ R-I), by stimulating G-protein activation (e.g.,  $\beta$ -adrenergic receptor), and by modulating associations with cytoplasmic signal transducing proteins (e.g., TNFR-1 and Fas/APO) (Heldin, Cell 80, 213-223 (1995)).

The tumor necrosis factor receptor (TNFR) superfamily is a group of type I transmembrane proteins which share a conserved cysteine-rich motif which is repeated three to six times in the extracellular domain (Smith, et al. Cell 76, 953-962 (1994)). Collectively, these repeat units form the ligand binding domains of these receptors (Chen et al., Chemistry 270, 2874-2878 (1995)). The ligands for these receptors are a structurally related group of proteins homologous to TNF $\alpha$ . (Goeddel et al. Cold Spring Harbor Symp. Quart. Biol. 51, 597-609 (1986); Nagata et al. Science 267, 1449-1456 (1995)). TNF $\alpha$  binds to distinct, but closely related receptors, TNFR-1 and TNFR-2. TNF $\alpha$  produces a variety of biological responses in receptor bearing cells, including, proliferation, differentiation, and cytotoxicity and apoptosis (Beutler et al. Ann. Rev. Biochem. 57, 505-518 (1988)).

TNF $\alpha$  is believed to mediate acute and chronic inflammatory responses (Beutler et al. Ann. Rev. Biochem. 57, 505-508 (1988)). Systemic delivery of TNF $\alpha$  induces toxic shock and widespread tissue necrosis. Because of this, TNF $\alpha$  may be responsible for the severe morbidity and mortality associated with a variety of infectious diseases, including sepsis. Mutations in FasL, the ligand for the TNFR-related receptor Fas/APO (Suda et al. Cell 75, 1169-1178 (1993)), is associated with autoimmunity (Fisher et al. Cell 81, 935-946 (1995)), while overproduction of FasL may be implicated

in drug-induced hepatitis. Thus, ligands to the various TNFR-related proteins often mediate the serious effects of many disease states, which suggests that agents that neutralize the activity of these ligands would have therapeutic value. Soluble TNFR-1 receptors, and antibodies that bind TNF $\alpha$ , have been tested for their ability to neutralize systemic TNF $\alpha$  (Loetscher et al. Cancer Cells 3(6), 221-226 (1991)). A naturally occurring form of a secreted TNFR-1 mRNA was recently cloned, and its product tested for its ability to neutralize TNF $\alpha$  activity in vitro and in vivo (Kohno et al. PNAS USA 87, 8331-8335 (1990)). The ability of this protein to neutralize TNF $\alpha$  suggests that soluble TNF receptors function to bind and clear TNF thereby blocking the cytotoxic effects on TNFR-bearing cells.

An object of the invention to identify new members of the TNFR super family. It is anticipated that new family members may be transmembrane proteins or soluble forms thereof comprising extracellular domains and lacking transmembrane and cytoplasmic domains. We have identified a new member of the TNFR superfamily which encodes a secreted protein that is closely related to TNFR-2. By analogy to soluble TNFR-1, the TNFR-2 related protein may negatively regulate the activity of its ligand, and thus may be useful in the treatment of certain human diseases.

#### Summary of the Invention

A novel member of the tumor necrosis factor receptor (TNFR) superfamily has been identified from a fetal rat intestinal cDNA library. A full-length cDNA clone was obtained and sequenced. Expression of the rat cDNA in a transgenic mouse revealed a marked increase in bones density, particularly in long bones, pelvic bone and vertebrae. The polypeptide encoded by

the cDNA is termed Osteoprotegerin (OPG) and plays a role in promoting bone accumulation.

5 The invention provides for nucleic acids encoding a polypeptide having at least one of the biological activities of OPG. Nucleic acids which hybridize to nucleic acids encoding mouse, rat or human OPG as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO: 122), and 9C-9D (SEQ ID NO: 124) are also provided. Preferably, OPG is mammalian OPG and more  
10 preferably is human OPG. Recombinant vectors and host cells expressing OPG are also encompassed as are methods of producing recombinant OPG. Antibodies or fragments thereof which specifically bind the polypeptide are also disclosed.

15 Methods of treating bone diseases are also provided by the invention. The polypeptides are useful for preventing bone resorption and may be used to treat any condition resulting in bone loss such as osteoporosis, hypercalcemia, Paget's disease of bone,  
20 and bone loss due to rheumatoid arthritis or osteomyelitis, and the like. Bone diseases may also be treated with anti-sense or gene therapy using nucleic acids of the invention. Pharmaceutical compositions comprising OPG nucleic acids and polypeptides are also  
25 encompassed.

#### Description of the Figures

30 Figure 1. A. FASTA analysis of novel EST LORF. Shown is the deduced FRI-1 amino acid sequence aligned to the human TNFR-2 sequence. B. Profile analysis of the novel EST LORF shown is the deduced FRI-1 amino acid sequence aligned to the TNFR-profile. C. Structural  
35 view of TNFR superfamily indicating region which is homologous to the novel FRI-1.

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Figure 2. Structure and sequence of full length rat OPG gene, a novel member of the TNFR superfamily. A. Map of pMOB-B1.1 insert. Box indicates position of LORF within the cDNA sequence (bold line). Black box indicates signal peptide, and gray ellipses indicate position of cysteine-rich repeat sequences. B, C. Nucleic acid and protein sequence of the Rat OPG cDNA. The predicted signal peptide is underlined, and potential sites of N-linked glycosylation are indicated in bold, underlined letters. D, E. Pileup sequence comparison (Wisconsin GCG Package, Version 8.1) of OPG with other members of the TNFR superfamily, fas (SEQ ID NO:128); tnfr1 (SEQ ID NO: 129); sfu-t2 (SEQ ID NO:130); tnfr2 (SEQ ID NO:131); cd40 (SEQ ID NO:132); osteo (SEQ ID NO:133); ngfr (SEQ ID NO:134); ox40 (SEQ ID NO:135); 41bb (SEQ ID NO:136).

Figure 3. PepPlot analysis (Wisconsin GCG Package, Version 8.1) of the predicted rat OPG protein sequence. A. Schematic representation of rat OPG showing hydrophobic (up) and hydrophilic (down) amino acids. Also shown are basic (up) and acidic (down) amino acids. B. Display of amino acid residues that are beta-sheet forming (up) and beta-sheet breaking down) as defined by Chou and Fasman (Adv. Enz. 47, 45-147 (1948)). C. Display of propensity measures for alpha-helix and beta-sheet (Chou and Fasman, *ibid*). Curves above 1.00 show propensity for alpha-helix or beta-sheet structure. Structure may terminate in regions of protein where curves drop below 1.00. D. Display of residues that are alpha-forming (up) or alpha-breaking (down). E. Display of portions of the protein sequence that resemble sequences typically found at the amino end of alpha and beta structures (Chou and Fasman, *ibid*). F. Display of portions of the protein sequence that resemble sequences typically found at the carboxyl

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end of alpha and beta structures (Chou and Fasman, ibid). G. Display of portions of the proteins sequence typically found in turns (Chou and Fasman, ibid) H. Display of the helical hydrophobic moment (Eisenberg et al. Proc. Natl. Acad. Sci. USA 81, 140-144 (1984)) at each position in the sequence. I. Display of average hydrophathy based upon Kyte and Doolittle (J. Mol. Biol. 157, 105-132 (1982)) and Goldman et al. (reviewed in Ann. Rev. Biophys. Biophys. Chem. 15, 321-353 (1986)).

Figure 4. mRNA expression patterns for the OPG cDNA in human tissues. Northern blots were probed with a 32P-labeled rat cDNA insert (A, left two panels), or with the human cDNA insert (B, right panel).

Figure 5. Creation of transgenic mice expressing the OPG cDNA in hepatocytes. Northern blot expression of HE-OPG transgene in mouse liver.

Figure 6. Increase in bone density in OPG transgenic mice. Panel A-F. Control Mice. G-J, OPG expressing mice. At necropsy, all animals were radiographed and photographs prepared. In A-F, the radiographs of the control animals and the one transgenic non-expressor (#28) are shown. Note that the bones have a clearly defined cortex and a lucent central marrow cavity. In contrast, the OPG (G-J) animals have a poorly defined cortex and increased density in the marrow zone.

Figure 7. Increase in trabecular bone in OPG transgenic mice. A-D. Representative photomicrographs of bones from control animals. In A and B, low (4X, 10X) power images of the femurs are shown (Masson Trichrome stain). Stains for tartrate resistant acid phosphatase (TRAP) demonstrate osteoclasts (see arrows)

both resorbing cartilage (C) and trabecular bone (D). Note the flattened appearance of osteoclasts on trabecular bone. E-H. Representative photomicrographs of bones from OPG-expressing animals.

5 In E and F, low (4X, 10X) power images of the femurs are shown (Masson Trichrome stain). The clear region is the growth plate cartilage, blue stained area is bone, and the red area is marrow. Note that in contrast to the controls, the trabecular bone has not been resorbed

10 resulting in the absence of the usual marrow cavity. Also, the resulting trabeculae have a variegated appearance with blue and clear areas. The clear areas are remnants of growth plate cartilage that have never been remodelled. Based on TRAP stains, these animals do

15 have osteoclasts (see arrows) at the growth plate (G), which may be reduced in number. However, the surfaces of the trabeculae away from the growth plate are virtually devoid of osteoclasts (H), a finding that stands in direct contrast with the control animals (see

20 D).

Figure 8. HE-OPG expressors do not have a defect in monocyte-macrophage development. One cause for osteopetrosis in mice is defective M-CSF production due

25 to a point mutation in the M-CSF gene. This results in a marked deficit of circulating and tissue based macrophages. The peripheral blood of OPG expressors contained monocytes as assessed by H1E analysis. To affirm the presence of tissue macrophages,

30 immunohistochemistry was performed using F480 antibodies, which recognize a cell surface antigen on murine macrophages. A and C show low power (4X) photomicrographs of the spleens from normal and CR1 overexpressors. Note that both animals have numerous

35 F480 positive cells. Monocyte-macrophages were also

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Figure 12. Structure of OPG cysteine-rich domains. Alignment of the human (top line SEQ ID NO:136) and mouse (bottom line) OPG amino acid sequences

highlighting the predicted domain structure of OPG. The polypeptide is divided into two halves; the N-terminus (A), and C-terminus (B). The N-terminal half is predicted to contain four cysteine rich domains (labeled 1-4). The predicted intrachain disulfide bonds are indicated by bold lines, labeled "SS1", "SS2", or "SS3". Tyrosine 28 and histidine 75 (underlined) are predicted to form an ionic interaction. Those amino acids predicted to interact with an OPG ligand are indicated by bold dots above the appropriate residue. The cysteine residues located in the C-terminal half of OPG are indicated by rectangular boxes.

Figure 13. Expression and secretion of full length and truncated mouse OPG-Fc fusion proteins. A. Map indicating points of fusion to the human IgG1 Fc domain are indicated by arrowheads. B. Silver stain of a SDS-polyacrylamide gel of conditioned media obtained from cells expressing either Fl.Fc (Full length OPG fused to Fc at Leucine 401) or CT.Fc (Carboxy-terminal truncated OPG fused to Fc at threonine 180) fusion protein expression vectors. Lane 1, parent pCEP4 expression vector cell line; Lane 2, Fl.Fc vector cell line; Lane 3, CT.Fc vector cell line. C. Western blot of conditioned media obtained from Fl.Fc and CT.Fc fusion protein expression vectors probed with anti-human IgG1 Fc domain (Pierce). Lane 1, parent pCEP4 expression vector cell line; Lane 2, Fl.Fc vector cell line; Lane 3, CT.Fc vector cell line.

Figure 14. Expression of human OPG in E. coli. A. Construction of a bacterial expression vector. The LORF of the human OPG gene was amplified by PCR, then joined to a oligonucleotide linker fragment (top strand is SEQ ID NO:137; bottom strand is SEQ ID NO:127), and

ligated into pAMG21 vector DNA. The resulting vector is capable of expressing OPG residues 32-401 linked to a N-terminal methionine residue. B SDS-PAGE analysis of uninduced and induced bacterial harboring the

5 pAMG21-human OPG -32-401 plasmid. Lane 1, MW standards; lane 2, uninduced bacteria; lane 3, 30°C induction; lane 4, 37°C induction; lane 5, whole cell lysate from 37°C induction; lane 6, soluble fraction of whole cell lysate; lane 7, insoluble fraction of whole

10 cell lysate; lane 8, purified inclusion bodies obtained from whole cell lysate.

Figure 15. Analysis of recombinant murine OPG produced in CHO cells by SDS-PAGE and western blotting. An

15 equal amount of CHO conditioned media was applied to each lane shown, and was prepared by treatment with either reducing sample buffer (left lane), or non-reducing sample buffer (right lane). After electrophoresis, the resolved proteins were transferred

20 to a nylon membrane, then probed with anti-OPG antibodies. The relative positions of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

25 Figure 16. ~~Pulse-chase analysis of recombinant murine OPG produced in CHO cells. CHO cells were pulse-labeled with <sup>35</sup>S-methionine/cysteine, then chased for the indicated time. Metabolically labeled cultures were separated into both conditioned media and cells, and detergent extracts were prepared from each, clarified, then immunoprecipitated with anti-OPG antibodies. The immunoprecipitates were resolved by SDS-PAGE, and exposed to film. Top left and right panels; samples analyzed under non-reducing conditions. Lower left and right panels; samples analyzed under reducing conditions. Top and bottom left panels; Cell~~

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extracts. Top and bottom right panels; Conditioned media extracts. The relative mobility of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

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Figure 17. Expression of OPG in the CTLL-2 cell line. Serum-free conditioned media from CTLL-2 cells and CHO-mu OPG [1-401] transfected cells was prepared, concentrated, then analyzed by non-reducing SDS-PAGE and western blotting. Left lane; CTLL-2 conditioned media. Right lane; CHO-muOPG conditioned media. The relative mobility of the 55 kd monomeric and 100 kd dimeric forms of OPG are indicated by arrowheads.

15 Figure 18. Detection of OPG expression in serum samples and liver extracts obtained from control and OPG transgenic mice. Transgenic mice were constructed as described in Example 4. OPG expression was visualized after SDS-PAGE followed by Western blotting using anti-OPG antibodies.

Figure 19. Effects of huOPG [22-401]-Fc fusion protein on osteoclast formation in vitro. The osteoclast forming assay was performed as described in Example 11A in the absence (control) or presence of the indicated amounts of huOPG [22-401]-Fc fusion. Osteoclast formation was visualized by histochemical staining for tartrate acid phosphatase (TRAP). ). A. OPG added to 100 ng/ml. D. OPG added to 0.1 ng/ml. E. OPG added to 0.01 ng/ml. F. OPG added to 0.001 ng/ml. G. Control. No OPG added.

Figure 20. Decrease in osteoclast culture TRAP activity with increasing amounts of OPG. Indicated concentrations of huOPG [22-401]-Fc fusion protein were

added to osteoclast forming assay and TRAP activity quantitated as described in Example 11A.

Figure 21. Effect of OPG on a terminal stage of osteoclast differentiation. huOPG [22-401]-Fc fusion was added to the osteoclast forming assay during the intermediate stage of osteoclast maturation (days 5-6; OPG-CTL) or during the terminal stage of osteoclast maturation (days 7-15; CTL-OPG). TRAP activity was quantitated and compared with the activity observed in the absence of OPG (CTL-CTL) in the presence of OPG throughout (OPG-OPG).

Figure 22. Effects of IL-1 $\beta$ , IL-1 $\alpha$  and OPG on blood ionized calcium in mice. Levels of blood ionized calcium were monitored after injection of IL-1 $\beta$  alone, IL-1 $\alpha$  alone, IL-1 $\beta$  plus muOPG [22-401]-Fc, IL-1 $\alpha$  plus muOPG [22-401]-Fc, and muOPG [22-401]-Fc alone. Control mice received injections of phosphate buffered saline (PBS) only. IL-1 $\beta$  experiment shown in A; IL-1 $\alpha$  experiment shown in B.

Figure 23. Effects of OPG on calvarial osteoclasts in control and IL1-treated mice. Histological methods for analyzing mice calvarial bone samples are described in Example 11B. Arrows indicate osteoclasts present in day 2-treated mice. Calvarial samples of mice receiving four PBS injections daily (A), one injection of IL-1 and three injections of PBS daily (B), one injection of PBS and three injections of OPG daily (C), one injection of IL-1 and three injections of OPG daily.

Figure 24. Radiographic analysis of bone accumulation in marrow cavity of normal mice. Mice were injected subcutaneously with saline (A) or muOPG [22-401]-Fc



fusion (5mg/kg/d) for 14 days (B) and bone density determined as described in Example 11C.

Figure 25. Histomorphometric analysis of bone accumulation in marrow cavity of normal mice. Injection experiments and bone histology performed as described in Example 11C.

Figure 26. Histology analysis of bone accumulation in marrow cavity of normal mice. Injection experiments and bone histology performed as described in Example 11C. A. Saline injection B. Injection of muOPG [22-401]-Fc fusion.

Figure 27. Activity of OPG administered to ovariectomized rats. In this two week experiment the trend to reduced bone density appears to be blocked by OPG or other anti-resorptive therapies. DEXA measurements were taken at time of ovariectomy and at week 1 and week 2 of treatment. The results are expressed as % change from the initial bone density (Mean +/- SEM).

Figure 28. Bone density in the femoral metaphysis, measured by histomorphometric methods, tends to be lower in ovariectomized rats (OVX) than sham operated animals (SHAM) 17 days following ovariectomy. This effect was blocked by OPG-Fc, with OPG-Fc treated ovariectomized rats (OVX+OPG) having significantly higher bone density than vehicle treated ovariectomized rats (OVX). (Mean +/- SEM).

#### Detailed Description of the Invention

A novel member of the tumor necrosis factor receptor (TNFR) superfamily was identified as an

expressed sequence tag (EST) isolated from a fetal rat intestinal cDNA library . The structures of the full-length rat cDNA clones and the corresponding mouse and human cDNA clones were determined as described in

5 Examples 1 and 6. The rat, mouse and human genes are shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124), respectively. All three sequences showed strong similarity to the extracellular domains of TNFR family members. None of

10 the full-length cDNA clones isolated encoded transmembrane and cytoplasmic domains that would be expected for membrane-bound receptors, suggesting that these cDNAs encode soluble, secreted proteins rather than cell surface receptors. A portion of the human

15 gene spanning nucleotides 1200-1353 shown in Figure 9D was deposited in the Genebank database on November 22, 1995 under accession no. 17188769.

The tissue distribution of the rat and human mRNA was determined as described in Example 2. In rat,

20 mRNA expression was detected in kidney, liver, placenta and heart with the highest expression in the kidney. Expression in skeletal muscle and pancreas was also detected. In humans, expression was detected in the same tissues along with lymph node, thymus, spleen and

25 appendix.

The rat cDNA was expressed in transgenic mice (Example 3) using the liver-specific ApoE promoter expression system. Analysis of expressors showed a marked increase in bone density, particularly in long

30 bones (femurs), vertebrae and flat bones (pelvis). Histological analysis of stained sections of bone showed severe osteopetrosis (see Example 4) indicating a marked imbalance between bone formation and resorption which has led to a marked accumulation of

35 bone and cartilage. A decrease in the number of trabecular osteoclasts in the bones of OPG expressor

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animals indicate that a significant portion of the activity of the TNFR-related protein may be to prevent bone resorption, a process mediated by osteoclasts. In view of the activity in transgenic expressors, the  
5 TNFR-related proteins described herein are termed OPGs.

Using the rat cDNA sequence, mouse and human cDNA clones were isolated (Example 5). Expression of mouse OPG in 293 cells and human OPG in E. coli is described in Examples 7 and 8. Mouse OPG was produced  
10 as an Fc fusion which was purified by Protein A affinity chromatography. Also described in Example 7 is the expression of full-length and truncated human and mouse OPG polypeptides in CHO and 293 cells either as fusion polypeptides to the Fc region of human IgG1  
15 or as unfused polypeptides. The expression of full-length and truncated human and mouse OPGs in E. coli either as Fc fusion polypeptides or as unfused polypeptides is described in Example 8. Purification of recombinantly produced mammalian and bacterial OPG  
20 is described in Example 10.

The biological activity of OPG was determined using an in vitro osteoclast maturation assay, an in vivo model of interleukin-1 (IL-1) induced hypercalcemia, and injection studies of bone density in  
25 normal mice (see Example 11). The following OPG recombinant proteins produced in CHO or 293 cells demonstrated activity in the in E. coli osteoclast maturation assay: muOPG [22-185]-Fc, muOPG [22-194]-Fc, muOPG [22-401]Fc, muOPG [22-401], huOPG [22-201]-Fc,  
30 huOPG [22-401]-Fc. muOPG [22-180]-Fc produced in CHO cells and huOPG met[32-401] produced in E. coli did not demonstrate activity in the in vitro assay.

OPG from several sources was produced as a dimer and to some extent as a higher multimer. Rat OPG  
35 [22-401] produced in transgenic mice, muOPG [22-401] and huOPG [22-401] produced as a recombinant

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polypeptide in CHO cells, and OPG expressed as a naturally occurring product from a cytotoxic T cell line were predominantly dimers and trimers when analyzed on nonreducing SDS gels (see Example 9).

- 5 Truncated OPG polypeptides having deletions in the region of amino acids 186-401 (e.g., OPG [1-185] and OPG [1-194]) were predominantly monomeric suggesting that the region 186-401 may be involved in self-association of OPG polypeptides. However, huOPG  
10 met[32-401] produced in E. coli was largely monomeric.

OPG may be important in regulating bone resorption. The protein appears to act as a soluble receptor of the TNF family and may prevent a receptor-ligand interaction involved in the osteolytic pathway.

- 15 One aspect of the regulation appears to be a reduction in the number of osteoclasts.

#### Nucleic Acids

- The invention provides for an isolated  
20 nucleic acid encoding a polypeptide having at least one of the biological activities of OPG. As described herein, the biological activities of OPG include, but are not limited to, any activity involving bone metabolism and in particular, include increasing bone  
25 density. The nucleic acids of the invention are selected from the following:

- a) the nucleic acid sequences as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124) or complementary strands thereof;  
30 b) the nucleic acids which hybridize under stringent conditions with the polypeptide-encoding region in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124); and  
c) nucleic acids which hybridize under stringent  
35 conditions with nucleotides 148 through 337 inclusive as shown in Figure 1A.

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d) the nucleic acid sequences which are degenerate to the sequences in (a) and (b).

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The invention provides for nucleic acids which encode rat, mouse and human OPG as well as

5 nucleic acid sequences hybridizing thereto which encode a polypeptide having at least one of the biological activities of OPG. Also provided for are nucleic acids which hybridize to a rat OPG EST encompassing

10 nucleotides 148-337 as shown in Figure 1A. The conditions for hybridization are generally of high stringency such as 5xSSC, 50% formamide and 42°C described in Example 1 of the specification. Equivalent stringency to these conditions may be

15 readily obtained by adjusting salt and organic solvent concentrations and temperature. The nucleic acids in (b) encompass sequences encoding OPG-related polypeptides which do not undergo detectable hybridization with other known members of the TNF receptor superfamily. In a preferred embodiment, the

20 nucleic acids are as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124).

The length of hybridizing nucleic acids of the invention may be variable since hybridization may

25 occur in part or all of the polypeptide-encoding regions as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124), and may also occur in adjacent noncoding regions. Therefore, hybridizing nucleic acids may be truncations or

30 extensions of the sequences shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124). Truncated or extended nucleic acids are encompassed by the invention provided they retain one or more of the biological properties of OPG. The

35 hybridizing nucleic acids may also include adjacent noncoding regions which are 5' and/or 3' to the OPG

coding region. The noncoding regions include regulatory regions involved in OPG expression, such as promoters, enhance, translational initiation sites, transcription termination sites and the like.

5           Hybridization conditions for nucleic acids are described in Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)

10           DNA encoding rat OPG was provided in plasmid pMO-B1.1 deposited with the American Type Culture Collection, Rockville, MD on December 27, 1995 under ATCC accession no. 69970. DNA encoding mouse OPG was provided in plasmid pRcCMV-murine OPG deposited with the American Type Culture Collection, Rockville, MD on  
15   December 27, 1995 under accession no. 69971. DNA encoding human OPG was provided in plasmid pRcCMV - human OPG deposited with the American Type Culture Collection, Rockville, MD on December 27, 1995 under accession no. 69969. The nucleic acids of the  
20   invention will hybridize under stringent conditions to the DNA inserts of ATCC accession nos. 69969, 69970, and 69971 and have at least one of the biological activities of OPG.

25           Also provided by the invention are derivatives of the nucleic acid sequences as shown in Figures 2B, 9A and 9B. As used herein, derivatives include nucleic acid sequences having addition, substitution, insertion or deletion of one or more residues such that the resulting sequences encode  
30   polypeptides having one or more amino acid residues which have been added, deleted, inserted or substituted and the resulting polypeptide has the activity of OPG. The nucleic acid derivatives may be naturally occurring, such as by splice variation or polymorphism,  
35   or may be constructed using site-directed mutagenesis techniques available to the skilled worker. One

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example of a naturally occurring variant of OPG is a nucleic acid encoding a lys to asn change at residue 3 within the leader sequence (see Example 5). It is anticipated that nucleic acid derivatives will encode amino acid changes in regions of the molecule which are least likely to disrupt biological activity. Other derivatives include a nucleic acid encoding a membrane-bound form of OPG having an extracellular domain as shown in Figures 2B-2C (SEQ ID NO:120), 9A-9B (SEQ ID NO:122), and 9C-9D (SEQ ID NO:124) along with transmembrane and cytoplasmic domains.

In one embodiment, derivatives of OPG include nucleic acids encoding truncated forms of OPG having one or more amino acids deleted from the carboxy terminus. Nucleic acids encoding OPG may have from 1 to 216 amino acids deleted from the carboxy terminus. Optionally, an antibody Fc region may extend from the new carboxy terminus to yield a biologically active OPG-Fc fusion polypeptide. (see Example 11). In preferred embodiments, nucleic acids encode OPG having the amino acid sequence from residues 22-185, 22-189, 22-194 or 22-201 (using numbering in Figure 9E-F) and optionally, encoding an Fc region of human IgG.

Also included are nucleic acids encoding truncated forms of OPG having one or more amino acids deleted from the amino terminus. Truncated forms include those lacking part or all the 21 amino acids comprising the leader sequence. Additionally, the invention provides for nucleic acids encoding OPG having from 1 to 10 amino acids deleted from the mature amino terminus (at residue 22) and ,optionally, having from 1 to 216 amino acids deleted from the carboxy terminus (at residue 401). Optionally, the nucleic acids may encode a methionine residue at the amino terminus. Examples of such OPG truncated polypeptides are described in Example 8.

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Examples of the nucleic acids of the invention include cDNA, genomic DNA, synthetic DNA and RNA. cDNA is obtained from libraries prepared from mRNA isolated from various tissues expressing OPG. In humans, tissue sources for OPG include kidney, liver, placenta and heart. Genomic DNA encoding OPG is obtained from genomic libraries which are commercially available from a variety of species. Synthetic DNA is obtained by chemical synthesis of overlapping oligonucleotide fragments followed by assembly of the fragments to reconstitute part or all of the coding region and flanking sequences (see U.S. Patent No. 4,695,623 describing the chemical synthesis of interferon genes). RNA is obtained most easily by procaryotic expression vectors which direct high-level synthesis of mRNA, such as vectors using T7 promoters and RNA polymerase.

Nucleic acid sequences of the invention are used for the detection of OPG sequences in biological samples in order to determine which cells and tissues are expressing OPG mRNA. The sequences may also be used to screen cDNA and genomic libraries for sequences related to OPG. Such screening is well within the capabilities of one skilled in the art using appropriate hybridization conditions to detect homologous sequences. The nucleic acids are also useful for modulating the expression of OPG levels by anti-sense therapy or gene therapy. The nucleic acids are also used for the development of transgenic animals which may be used for the production of the polypeptide and for the study of biological activity (see Example 3).

#### Vectors and Host Cells

Expression vectors containing nucleic acid sequences encoding OPG, host cells transformed with

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5 said vectors and methods for the production of OPG are also provided by the invention. An overview of expression of recombinant proteins is found in Methods of Enzymology v. 185, Goeddel, D.V. ed. Academic Press (1990).

Host cells for the production of OPG include procaryotic host cells, such as E. coli, yeast, plant, insect and mammalian host cells. E. coli strains such as HB101 or JM101 are suitable for expression.

10 Preferred mammalian host cells include COS, CHOd-, 293, CV-1, 3T3, baby hamster kidney (BHK) cells and others. Mammalian host cells are preferred when post-translational modifications, such as glycosylation and polypeptide processing, are important for OPG activity.

15 Mammalian expression allows for the production of secreted polypeptides which may be recovered from the growth medium.

Vectors for the expression of OPG contain at a minimum sequences required for vector propagation and

20 for expression of the cloned insert. These sequences include a replication origin, selection marker, promoter, ribosome binding site, enhancer sequences, RNA splice sites and transcription termination site. Vectors suitable for expression in the aforementioned

25 host cells are readily available and the nucleic acids of the invention are inserted into the vectors using standard recombinant DNA techniques. Vectors for tissue-specific expression of OPG are also included. Such vectors include promoters which function

30 specifically in liver, kidney or other organs for production in mice, and viral vectors for the expression of OPG in targeted human cells.

Using an appropriate host-vector system, OPG is produced recombinantly by culturing a host cell

35 transformed with an expression vector containing nucleic acid sequences encoding OPG under conditions

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The invention also provides for expression of OPG from endogenous nucleic acids by in vivo or ex vivo recombination events to allow modulation of OPG from the host chromosome. Expression of OPG by the introduction of exogenous regulatory sequences (e.g. promoters or enhancers) capable of directing the production of OPG from endogenous OPG coding regions is also encompassed. Stimulation of endogenous regulatory sequences capable of directing OPG production (e.g. by exposure to transcriptional enhancing factors) is also provided by the invention.

35           The invention provides for OPG, a novel  
member of the TNF receptor superfamily, having an

activity associated with bone metabolism and in particular having the activity of inhibiting bone resorption thereby increasing bone density. OPG refers to a polypeptide having an amino acid sequence of mouse, rat or human OPG or a derivative thereof having at least one of the biological activities of OPG. The amino acid sequences of rat, mouse and human OPG are shown in Figures 2B-2C (SEQ ID NO:121), 9A-9B (SEQ ID NO:123), and 9C-9D (SEQ ID NO:125) respectively. A derivative of OPG refers to a polypeptide having an addition, deletion, insertion or substitution of one or more amino acids such that the resulting polypeptide has at least one of the biological activities of OPG. The biological activities of OPG include, but are not limited to, activities involving bone metabolism. Preferably, the polypeptides will have the amino terminal leader sequence of 21 amino acids removed.

OPG polypeptides encompassed by the invention include rat [1-401], rat [22-180], rat [22-401], rat [22-401]-Fc fusion, rat [1-180]-Fc fusion, mouse [1-401], mouse [1-180], mouse [22-401], human [1-401], mouse [22-180], human [22-401], human [22-180], human [1-180], human [22-180]-Fc fusion and human met-32-401. Amino acid numbering is as shown in SEQ ID NO:121 (rat), SEQ ID NO:123 (mouse) and SEQ ID NO:125 (human). Also encompassed are polypeptide derivatives having deletions or carboxy-terminal truncations of part or all of amino acids residues 180-401 of OPG; one or more amino acid changes in residues 180-401; deletion of part or all of a cysteine-rich domain of OPG, in particular deletion of the distal (carboxy-terminal) cysteine-rich domain; and one or more amino acid changes in a cysteine-rich domain, in particular in the distal (carboxy-terminal) cysteine-rich domain. In one embodiment, OPG has from 1 to about 216 amino acids deleted from the carboxy terminus. In another

embodiment, OPG has from 1 to about 10 amino acids deleted from the mature amino terminus (wherein the mature amino terminus is at residue 22) and, optionally, has from 1 to about 216 amino acids deleted from the carboxy terminus.

Additional OPG polypeptides encompassed by the invention include the following: human [22-180]-Fc fusion, human [22-201]-Fc fusion, human [22-401]-Fc fusion, mouse [22-185]-Fc fusion, mouse [22-194]-Fc fusion. These polypeptides are produced in mammalian host cells, such as CHO or 293 cells, Additional OPG polypeptides encompassed by the invention which are expressed in procaryotic host cells include the following: human met[22-401], Fc-human met[22-401] fusion (Fc region is fused at the amino terminus of the full-length OPG coding sequence as described in Example 8), human met[22-401]-Fc fusion (Fc region fused to the full-length OPG sequence), Fc-mouse met[22-401] fusion, mouse met[22-401]-Fc fusion, human met[27-401], human met[22-185], human met[22-189], human met[22-194], human met[22-194] (P25A), human met [22-194] (P26A), human met[27-185], human met[27-189], human met[27-194], human met-arg-gly-ser-(his)<sub>6</sub> [22-401], human met-lys [22-401], human met-(lys)<sub>3</sub>-[22-401], human met[22-401]-Fc (P25A), human met[22-401] (P25A), human met[22-401] (P26A), human met[22-401] (P26D), mouse met[22-401], mouse met[27-401], mouse met[32-401], mouse met[27-180], mouse met[22-189], mouse met[22-194], mouse met[27-189], mouse met[27-194], mouse met-lys[22-401], mouse HEK[22-401] (A45T), mouse met-lys-(his)<sub>7</sub>[22-401], mouse met-lys[22-401]-(his)<sub>7</sub> and mouse met[27-401] (P33E, G36S, A45P). It is understood that the above OPG polypeptides produced in procaryotic host cells have an amino-terminal methionine residue, if such a residue is not indicated. In specific examples, OPG-Fc fusion were produced using a 227 amino

acid region of human IgG1- $\gamma$ 1 was used having the sequence as shown in Ellison et al. (Nuc. Acids Res. 10, 4071-4079 (1982)). However, variants of the Fc region of human IgG may also be used.

5           Analysis of the biological activity of carboxy-terminal OPG truncations fused to the human IgG1 Fc region indicates a portion of OPG of about 164 amino acids which is required for activity. This region encompasses amino acids 22-185, preferably those  
10 in Figure 9C-9D (SEQ ID NO:125), and comprises four cysteine-rich domains characteristic of the cysteine-rich domains of TNFR extraceullular domains.

          Using the homology between OPG and the extracellular ligand binding domains of TNF receptor  
15 family members, a three-dimensional model of OPG was generated based upon the known crystal structure of the extracellular domain of TNFR-I (see Example 6). This model was used to identify those residues within OPG which may be important for biological activity.  
20 Cysteine residues that are involved in maintaining the structure of the four cysteine-rich domains were identified. The following disulfide bonds were identified in the model: Domain 1: cys41 to cys54, cys44 to cys62, tyr23 and his 66 may act to stabilize  
25 the structure of this domain; Domain 2: cys65 to cys80, cys83 to cys98, cys87 to cys105; Domain 3: cys107 to cys118, cys124 to cys142; Domain 4: cys145 to cys160, cys166 to cys185. Residues were also identified which were in close proximity to TNF $\beta$  as shown in Figures 11  
30 and 12A-12B. In this model, it is assumed that OPG binds to a corresponding ligand; TNF $\beta$  was used as a model ligand to simulate the interaction of OPG with its ligand. Based upon this modeling, the following residues in OPG may be important for ligand binding:  
35 glu34, lys43, pro66 to gln91 (in particular, pro66,

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his68, tyr69, tyr70, thr71, asp72, ser73, his76, ser77, asp78, glu79, leu81, tyr82, pro85, val86, lys88, glu90 and gln91), glu153 and ser155.

Alterations in these amino acid residues, either singly or in combination, may alter the biological activity of OPG. For example, changes in specific cysteine residues may alter the structure of individual cysteine-rich domains, whereas changes in residues important for ligand binding may affect physical interactions of OPG with ligand. Structural models can aid in identifying analogs which have more desirable properties, such as enhanced biological activity, greater stability, or greater ease of formulation.

The invention also provides for an OPG multimer comprising OPG monomers. OPG appears to be active as a multimer (e.g, dimer, trimer of a higher number of monomers). Preferably, OPG multimers are dimers or trimers. OPG multimers may comprise monomers having the amino acid sequence of OPG sufficient to promote multimer formation or may comprise monomers having heterologous sequences such as an antibody Fc region. Analysis of carboxy-terminal deletions of OPG suggest that at least a portion of the region 186-401 is involved in association of OPG polypeptides. Substitution of part or all of the region of OPG amino acids 186-401 with an amino acid sequence capable of self-association is also encompassed by the invention. Alternatively, OPG polypeptides or derivatives thereof may be modified to form dimers or multimers by site directed mutagenesis to create unpaired cysteine residues for interchain disulfide bond formation, by photochemical crosslinking, such as exposure to ultraviolet light, or by chemical crosslinking with bifunctional linker molecules such as bifunctional polyethylene glycol and the like.

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Modifications of OPG polypeptides are encompassed by the invention and include post-translational modifications (e.g., N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition of an N-terminal methionine residue as a result of procaryotic host cell expression. The polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

Further modifications of OPG include chimeric proteins wherein OPG is fused to a heterologous amino acid sequence. The heterologous sequence may be any sequence which allows the resulting fusion protein to retain the activity of OPG. The heterologous sequences include for example, immunoglobulin fusions, such as Fc fusions, which may aid in purification of the protein. A heterologous sequence which promotes association of OPG monomers to form dimers, trimers and other higher multimeric forms is preferred.

The polypeptides of the invention are isolated and purified from other polypeptides present in tissues, cell lines and transformed host cells expressing OPG, or purified from components in cell cultures containing the secreted protein. In one embodiment, the polypeptide is free from association with other human proteins, such as the expression product of a bacterial host cell.

Also provided by the invention are chemically modified derivatives of OPG which may provide additional advantages such as increasing stability and circulating time of the polypeptide, or decreasing immunogenicity (see U.S. Patent No. 4,179,337). The chemical moieties for derivitization may be selected

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chemical crosslinkers may be used depending upon which properties of the protein dimer are desired. For example, crosslinkers may be short and relatively rigid or longer and more flexible, may be biologically  
5 reversible, and may provide reduced immunogenicity or longer pharmacokinetic half-life.

In one example, OPG molecules are linked through the amino terminus by a two step synthesis (see Example 12). In the first step, OPG is chemically  
10 modified at the amino terminus to introduce a protected thiol, which after purification is deprotected and used as a point of attachment for site-specific conjugation through a variety of crosslinkers with a second OPG molecule. Amino-terminal crosslinks include, but are  
15 not limited to, a disulfide bond, thioether linkages using short-chain, bis-functional aliphatic crosslinkers, and thioether linkages to variable length, bifunctional polyethylene glycol crosslinkers (PEG "dumbbells"). Also encompassed by PEG dumbbell  
20 synthesis of OPG dimers is a byproduct of such synthesis, termed a "monobell". An OPG monobell consists of a monomer coupled to a linear bifunctional PEG with a free polymer terminus. Alternatively, OPG may be crosslinked directly through a variety of amine  
25 specific homobifunctional crosslinking techniques which include reagents such as: diethylenetriaminepentaacetic dianhydride (DTPA), p-benzoquinone (pBQ) or bis(sulfosuccinimidyl) suberate (BS<sup>3</sup>) as well as others known in the art. It is also possible to thiolate OPG  
30 directly with reagents such as iminothiolane in the presence of a variety of bifunctional, thiol specific crosslinkers, such as PEG bismaleimide, and achieve dimerization and/or dumbbells in a one step process.

A method for the purification of OPG from  
35 natural sources and from transfected host cells is also included. The purification process may employ one or

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more standard protein purification steps in an appropriate order to obtain purified protein. The chromatography steps can include ion exchange, gel filtration, hydrophobic interaction, reverse phase, chromatofocusing, affinity chromatography employing an anti-OPG antibody or biotin-streptavidin affinity complex and the like.

### Antibodies

Also encompassed by the invention are antibodies specifically binding to OPG. Antigens for the generation of antibodies may be full-length polypeptides or peptides spanning a portion of the OPG sequence. Immunological procedures for the generation of polyclonal or monoclonal antibodies reactive with OPG are known to one skilled in the art (see, for example, Harlow and Lane, Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor N.Y. (1988)). Antibodies so produced are characterized for binding specificity and epitope recognition using standard enzyme-linked immunosorbent assays. Antibodies also include chimeric antibodies having variable and constant domain regions derived from different species. In one embodiment, the chimeric antibodies are humanized antibodies having murine variable domains and human constant domains. Also encompassed are complementary determining regions grafted to a human framework (so-called CDR-grafted antibodies). Chimeric and CDR-grafted antibodies are made by recombinant methods known to one skilled in the art. Also encompassed are human antibodies made in mice.

Anti-OPG antibodies of the invention may be used as an affinity reagent to purify OPG from biological samples (see Example 10). In one method, the antibody is immobilized on CnBr-activated Sepharose

and a column of antibody-Sepharose conjugate is used to remove OPG from liquid samples. Antibodies are also used as diagnostic reagents to detect and quantitate OPG in biological samples by methods described below.

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Pharmaceutical compositions

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the polypeptide of the invention together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. The term "therapeutically effective amount" means an amount which provides a therapeutic effect for a specified condition and route of administration. The composition may be in a liquid or lyophilized form and comprises a diluent (Tris, acetate or phosphate buffers) having various pH values and ionic strengths, solubilizer such as Tween or Polysorbate, carriers such as human serum albumin or gelatin, preservatives such as thimerosal or benzyl alcohol, and antioxidants such as ascorbic acid or sodium metabisulfite. Also encompassed are compositions comprising OPG modified with water soluble polymers to increase solubility or stability.

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Specifically, OPG compositions may comprise incorporation into polymer matrices such as hydrogels, silicones, polyethylenes, ethylene-vinyl acetate copolymers, or biodegradable polymers. Examples of hydrogels include polyhydroxyalkylmethacrylates (p-HEMA), polyacrylamide, polymethacrylamide, polyvinylpyrrolidone, polyvinyl alcohol and various polyelectrolyte complexes. Examples of biodegradable

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polymers include polylactic acid (PLA), polyglycolic acid (PGA), copolymers of PLA and PGA, polyamides and copolymers of polyamides and polyesters. Other controlled release formulations include microcapsules, microspheres, macromolecular complexes and polymeric beads which may be administered by injection.

Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration and the pharmacokinetic parameters desired. A more extensive survey of component suitable for pharmaceutical compositions is found in Remington's Pharmaceutical Sciences, 18th ed. A.R. Gennaro, ed. Mack, Easton, PA (1980).

Compositions of the invention may be administered by injection, either subcutaneous, intravenous or intramuscular, or by oral, nasal, pulmonary or rectal administration. The route of administration eventually chosen will depend upon a number of factors and may be ascertained by one skilled in the art.

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the nucleic acids of the invention together with a pharmaceutically acceptable adjuvant. Nucleic acid compositions will be suitable for the delivery of part or all of the OPG coding region to cells and tissues as part of an anti-sense or gene therapy regimen.

#### Methods of Treatment

Bone tissue provides support for the body and consists of mineral (largely calcium and phosphorous), a matrix of collagenous and noncollagenous proteins, and cells. Three types of cells found in bone, osteocytes, osteoblasts and osteoclasts, are involved

in the dynamic process by which bone is continually formed and resorbed. Osteoblasts promote formation of bone tissue whereas osteoclasts are associated with resorption. Resorption, or the dissolution of bone matrix and mineral, is a fast and efficient process compared to bone formation and can release large amounts of mineral from bone. Osteoclasts are involved in the regulation of the normal remodeling of skeletal tissue and in resorption induced by hormones. For instance, resorption is stimulated by the secretion of parathyroid hormone in response to decreasing concentrations of calcium ion in extracellular fluids. In contrast, inhibition of resorption is the principal function of calcitonin. In addition, metabolites of vitamin D alter the responsiveness of bone to parathyroid hormone and calcitonin.

After skeletal maturity, the amount of bone in the skeleton reflects the balance (or imbalance) of bone formation and bone resorption. Peak bone mass occurs after skeletal maturity prior to the fourth decade. Between the fourth and fifth decades, the equilibrium shifts and bone resorption dominates. The inevitable decrease in bone mass with advancing years starts earlier in females than males and is distinctly accelerated after menopause in some females (principally those of Caucasian and Asian descent).

Osteopenia is a condition relating generally to any decrease in bone mass to below normal levels. Such a condition may arise from a decrease in the rate of bone synthesis or an increase in the rate of bone destruction or both. The most common form of osteopenia is primary osteoporosis, also referred to as postmenopausal and senile osteoporosis. This form of osteoporosis is a consequence of the universal loss of bone with age and is usually a result of increase in bone resorption with a normal rate of bone formation.

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About 25 to 30 percent of all white females in the United States develop symptomatic osteoporosis. A direct relationship exists between osteoporosis and the incidence of hip, femoral, neck and inter-trochanteric fracture in women 45 years and older. Elderly males develop symptomatic osteoporosis between the ages of 50 and 70, but the disease primarily affects females.

The cause of postmenopausal and senile osteoporosis is unknown. Several factors have been identified which may contribute to the condition. They include alteration in hormone levels accompanying aging and inadequate calcium consumption attributed to decreased intestinal absorption of calcium and other minerals. Treatments have usually included hormone therapy or dietary supplements in an attempt to retard the process. To date, however, an effective treatment for bone loss does not exist.

The invention provides for a method of treating a bone disorder using a therapeutically effective amount of OPG. The bone disorder may be any disorder characterized by a net bone loss (osteopenia or osteolysis). In general, treatment with OPG is anticipated when it is necessary to suppress the rate of bone resorption. Thus treatment may be done to reduce the rate of bone resorption where the resorption rate is above normal or to reduce bone resorption to below normal levels in order to compensate for below normal levels of bone formation.

Conditions which are treatable with OPG include the following:

Osteoporosis, such as primary osteoporosis, endocrine osteoporosis (hyperthyroidism, hyperparathyroidism, Cushing's syndrome, and acromegaly), hereditary and congenital forms of osteoporosis (osteogenesis imperfecta, homocystinuria,

Menkes' syndrome, and Riley-Day syndrome) and osteoporosis due to immobilization of extremities.

    Paget's disease of bone (osteitis deformans) in adults and juveniles

5           Osteomyelitis, or an infectious lesion in bone, leading to bone loss.

          Hypercalcemia resulting from solid tumors (breast, lung and kidney) and hematologic malignancies (multiple myeloma, lymphoma and leukemia), idiopathic  
10 hypercalcemia, and hypercalcemia associated with hyperthyroidism and renal function disorders.

          Osteopenia following surgery, induced by steroid administration, and associated with disorders of the small and large intestine and with chronic  
15 hepatic and renal diseases.

          Osteonecrosis, or bone cell death, associated with traumatic injury or nontraumatic necrosis associated with Gaucher's disease, sickle cell anemia, systemic lupus erythematosus and other conditions.

20           Bone loss due to rheumatoid arthritis.

          Periodontal bone loss.

          Osteolytic metastasis

          It is understood that OPG may be used alone or in conjunction with other factors for the treatment  
25 of bone disorders. In one embodiment, osteoprotegerin is used in conjunction with a therapeutically effective amount of a factor which stimulates bone formation. Such factors include but are not limited to the bone morphogenic factors designated BMP-1 through BMP-12,  
30 transforming growth factor- $\beta$  (TGF- $\beta$ ) and TGF- $\beta$  family members, interleukin-1 inhibitors, TNF $\alpha$  inhibitors, parathyroid hormone and analogs thereof, parathyroid related protein and analogs thereof, E series prostaglandins, bisphosphonates (such as alendronate  
35 and others), and bone-enhancing minerals such as fluoride and calcium.

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The following examples are offered to more fully illustrate the invention, but are not construed as limiting the scope thereof.

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#### EXAMPLE 1

##### Identification and isolation of the rat OPG cDNA

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Materials and methods for cDNA cloning and analysis are described in Maniatis et al, ibid. Polymerase chain reactions (PCR) were performed using a  
15 Perkin-Elmer 9600 thermocycler using PCR reaction mixture (Boehringer-Mannheim) and primer concentrations specified by the manufacturer. In general, 25-50  $\mu$ l reactions were denatured at 94°C, followed by 20-40 cycles of 94°C for 5 seconds, 50-60°C for 5 seconds,  
20 and 72°C for 3-5 minutes. Reactions were then treated for 72 °C for 3-5 minutes. Reactions were then analyzed by gel electrophoresis as described in Maniatis et al., ibid.

A cDNA library was constructed using mRNA  
25 isolated from embryonic d20 intestine for EST analysis (Adams et al. Science 252, 1651-1656 (1991)). Rat embryos were dissected, and the entire developing small and large intestine removed and washed in PBS. Total cell RNA was purified by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi  
30 Anal. Biochem. 162, 156-159, (1987)). The poly (A+) mRNA fraction was obtained from the total RNA preparation by adsorption to, and elution from, Dynabeads Oligo (dT)25 (Dynal Corp) using the  
35 manufacturer's recommended procedures. A random primed cDNA library was prepared using the Superscript Plasmid

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System (Gibco BRL, Gaithersburg, Md). The random cDNA primer containing an internal Not I restriction site was used to initiate first strand synthesis and had the following sequence:

5 5'-AAAGGAAGGAAAAAAGCGGCCGCTACANNNNNNNNT-3' (SEQ ID NO:1)

#### Not I

For the first strand synthesis three separate reactions were assembled that contained 2.5 µg of  
10 poly(A) RNA and 120 ng, 360 ng or 1,080 ng of random primer. After second strand synthesis, the reaction products were separately extracted with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1 ratio), and then ethanol precipitated. The double strand (ds) cDNA  
15 products of the three reactions were combined and ligated to the following ds oligonucleotide adapter:

5'-TCGACCCACGCGTCCG-3' (SEQ ID NO:2)

3'-GGGTGCGCAGGCp-5' (SEQ ID NO:3)

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After ligation the cDNA was digested to completion with Not I, extracted with phenol:chloroform:isoamyl (25:24:1) alcohol and ethanol precipitated. The resuspended cDNA was then size  
25 fractionated by gel filtration using premade columns provided with the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md) as recommended by the manufacturer. The two fractions containing the largest cDNA products were pooled, ethanol precipitated and  
30 then directionally ligated into Not I and Sal I digested pMOB vector DNA (Strathmann et al, 1991). The ligated cDNA was introduced into competent ElectroMAX DH10B E. coli (Gibco BRL, Gaithersburg, MD) by electroporation. For automated sequence analysis  
35 approximately 10,000 transformants were plated on 20cm x 20cm agar plates containing ampicillin supplemented

LB nutrient media. The colonies that arose were picked and arrayed onto 96 well microtiter plates containing 200 µl of L-broth, 7.5% glycerol, and 50 µg/ml ampicillin. The cultures were grown overnight at 37°C, a duplicate set of microtiter plates were made using a sterile 96 pin replicating tool, then both sets were stored at -80°C for further analysis. For full-length cDNA cloning approximately one million transformants were plated on 96 bacterial ampicillin plates containing about 10,000 clones each. The plasmid DNA from each pool was separately isolated using the Qiagen Plasmid Maxi Kit (Qiagen Corp., Germany) and arrayed into 96 microtiter plates for PCR analyses.

To sequence random fetal rat intestine cDNA clones, glycerol stocks were thawed, and small aliquots diluted 1:25 in distilled. Approximately 3.0 µl of diluted bacterial cultures were added to PCR reaction mixture (Boehringer-Mannheim) containing the following oligonucleotides:

5'-TGTAACGACGGCCAGT-3' (SEQ ID NO:4)

5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO:5)

The reactions were incubated in a thermocycler (Perkin-Elmer 9600) with the following cycle conditions: 94°C for 2 minutes; 30 cycles of 94°C for 5 seconds, 50°C for 5 seconds, and 72°C for 3 minutes.; 72°C for 4 minutes. After incubation in the thermocycler, the reactions were diluted with 2.0 mL of water. The amplified DNA fragments were further purified using Centricon columns (Princeton Separations) using the manufacturer's recommended procedures. The PCR reaction products were sequenced on an Applied Biosystems 373A automated DNA sequencer using T3 primer (oligonucleotide 353-23; 5'-CAATTAACCCTCACTAAAGG-3') (SEQ ID NO:6) Taq dye-

The resulting 5' nucleotide sequence obtained from randomly picked cDNA clones translated and then compared to the existing database of known protein sequences using a modified version of the FASTA program (Pearson et al. Meth. Enzymol. 183, (1990)).

Translated sequences were also analysed for the presence of a specific cysteine-rich protein motif found in all known members of the tumor necrosis factor receptor (TNFR) superfamily (Smith et al. Cell 76, 959-962 (1994)), using the sequence profile method of Gribskov et al. (Proc. Natl. Acad. Sci. USA 83, 4355-4359 (1987)), as modified by Luethy et al.

Using the FASTA and Profile search data, an EST, FRI-1 (Fetal Rat Intestine-1), was identified as a possible new member of the TNFR superfamily. FRI-1 contained an approximately 600 bp insert with a LORF of about 150 amino acids. The closest match in the database was the human type II TNFR (TNFR-2). The region compared showed an ~43% homology between TNFR-2 and FRI-1 over this 150 aa LORF. Profile analysis using the first and second cysteine-rich repeats of the TNFR superfamily yielded a Z score of ~8, indicating that the FRI-1 gene possibly encodes a new family member.

To deduce the structure of the FRI-1 product, the fetal rat intestine cDNA library was screened for full length clones. The following oligonucleotides were derived from the original FRI-1 sequence:

5'-AGGTAGCGCCCTTCCTCACATTC-3' (SEQ ID NO:8)

These primers were used in PCR reactions to screen 96 pools of plasmid DNA, each pool containing plasmid DNA from 10,000 independent cDNA clones. Approximately 1 ug of plasmid pool DNA was amplified in a PCR reaction mixture (Boehringer-Mannheim) using a Perkin-Elmer 96 well thermal cyclor with the following cycle conditions: 2 min at 94°C, 1 cycle; 15 sec at 94°C, then 45 sec at 65°C, 30 cycles; 7 min at 65°C, 1 cycle. PCR reaction products were analysed by gel electrophoresis. 13 out of 96 plasmid DNA pools gave rise to amplified DNA products with the expected relative molecular mass.

DNA from one positive pool was used to transform competent ElectroMAX DH10B E. coli (Gibco BRL, Gaithersburg, MD) as described above. Approximately 40,000 transformants were plated onto sterile nitrocellulose filters (BA-85, Schleicher and Schuell), and then screened by colony hybridization using a <sup>32</sup>P-dCTP labelled version of the PCR product obtained above. Filters were prehybridized in 5X SSC, 50% deionized formamide, 5X Denhardt's solution, 0.5% SDS, and 100 ug/ml denatured salmon sperm DNA for 2-4 hours at 42°C. Filters were then hybridized in 5X SSC, 50% deionized formamide, 2X Denhardt's solution, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA, and ~5 ng/ml of labelled probe for ~18 hours at 42°C. The filters were then washed in 2X SSC for 10 min at RT, 1X SSC for 10 min at 55°C, and finally in 0.5X SSC for 10-15 min at 55°C. Hybridizing clones were detected following autoradiography, and then replated onto nitrocellulose filters for secondary screening. Upon secondary screening, a plasmid clone (pB1.1) was isolated, then amplified in L-broth media containing 100 ug/ml ampicillin and the plasmid DNA obtained. Both strands of the 2.4 kb pB1.1 insert were sequenced.

The pB1.1 insert sequence was used for a FASTA search of the public database to detect any existing sequence matches and/or similarities. No matches to any known genes or EST's were found, although there was an approximate 45% similarity to the human and mouse TNFR-2 genes. A methionine start codon is found at bp 124 of the nucleotide sequence, followed by a LORF encoding 401 aa residues that terminates at bp 1327. The 401 aa residue product is predicted to have a hydrophobic signal peptide of approximately 31 residues at its N-terminus, and 4 potential sites of N-linked glycosylation. No hydrophobic transmembrane spanning sequence was identified using the PepPlot program (Wisconsin GCG package, version 8.1). The deduced 401 aa sequence was then used to search the protein database. Again, there were no existing matches, although there appeared to be a strong similarity to many members of the TNFR superfamily, most notably the human and mouse TNFR-2. A sequence alignment of this novel protein with known members of the TNFR-superfamily was prepared using the Pileup program, and then modified by PrettyPlot (Wisconsin GCG package, version 8.1). This alignment shows a clear homology between the full length FRI-1 gene product and all other TNFR family members. The homologus region maps to the extracellular domain of TNFR family members, and corresponds to the three or four cysteine-rich repeats found in the ligand binding domain of these proteins. This suggested that the FRI-1 gene encoded a novel TNFR family member. Since no transmembrane spanning region was detected we predicted that this may be a secreted receptor, similar to TNFR-1 derived soluble receptors (Kohno et al. Proc. Natl. Acad. Sci. USA 87, 8331-8335 (1990)). Due to the apparent biological activity of the FRI-1 gene (*vide infra*), the product was named Osteoprotegerin (OPG).

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## EXAMPLE 2

### OPG mRNA Expression Patterns in Tissues

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Multiple human tissue northern blots (Clonetech) were probed with a  $^{32}\text{P}$ -dCTP labelled FRI-1 PCR product to detect the size of the human transcript and to determine patterns of expression. Northern  
10 blots were prehybridized in 5X SSPE, 50% formamide, 5X Denhardt's solution, 0.5% SDS, and 100  $\mu\text{g}/\text{ml}$  denatured salmon sperm DNA for 2-4 hr at 42°C. The blots were then hybridized in 5X SSPE, 50% formamide, 2X Denhardt's solution, 0.1% SDS, 100  $\mu\text{g}/\text{ml}$  denatured  
15 salmon sperm DNA, and 5 ng/ml labelled probe for 18-24 hr at 42°C. The blots were then washed in 2X SSC for 10 min at RT, 1X SSC for 10 min at 50°C, then in 0.5X SSC for 10-15 min.

Using a probe derived from the rat gene, a  
20 predominant mRNA species with a relative molecular mass of about 2.4 kb is detected in several tissues, including kidney, liver, placenta, and heart. Highest levels are detected in the kidney. A large mRNA species of Mr 4.5 and 7.5 kb was detected in skeletal  
25 muscle and pancreas. In human fetal tissue, kidney was found to express relatively high levels of the 2.4 kb mRNA. Using a human probe (vide infra), only the 2.4 kb transcript is detected in these same tissues. In addition, relatively high levels of the 2.4 kb  
30 transcript was detected in the lymph node, thymus, spleen and appendix. The size of the transcript detected by both the rat and human Osteosprotegerin gene is almost identical to the length of the rat pB1.1 FRI-1 insert, suggesting it was a full length cDNA  
35 clone.

EXAMPLE 3

Systemic delivery of OPG in transgenic mice

The rat OPG clone pB1.1 was used as template  
5 to PCR amplify the coding region for subcloning into an  
ApoE-liver specific expression vector (Simonet et al.  
J. Clin. Invest. 94, 1310-1319 (1994), and PCT  
Application No. US94/11675 and co-owned U.S. Serial No.  
08/221,767. The following 5' and 3' oligonucleotide  
10 primers were used for PCR amplification, respectively:

5'-GACTAGTCCCACAATGAACAAGTGGCTGTG-3' (SEQ ID NO:9)  
5'-ATAAGAATGCGGCCGCTAAACTATGAAACAGCCCAGTGACCATTTC-3'  
(SEQ ID NO:10)

15

The PCR reaction mixture (Boehringer-  
Mannheim) was treated as follows: 94°C for 1 minute, 1  
cycle; 94°C for 20 sec, 62°C for 30 sec, and 74 C for 1  
minute, 25 cycles. Following amplification, the  
20 samples were purified over Qiagen PCR columns and  
digested overnight with SpeI and NotI restriction  
enzymes. The digested products were extracted and  
precipitated and subcloned into the ApoE promoter  
expression vector. Prior to microinjecting the  
25 resulting clone, HE-OPG, it was sequenced to ensure it  
was mutation-free.

The HE-OPG plasmid was purified through two  
rounds of CsCl density gradient centrifugation. The  
purified plasmid DNA was digested with XhoI and Ase I,  
30 and the 3.6 kb transgene insert was purified by gel  
electrophoresis. The purified fragment was diluted to  
a stock injection solution of 1 µg/ml in 5 mM Tris, pH  
7.4, 0.2 mM EDTA. Single-cell embryos from BDF1 x  
BDF1-bred mice were injected essentially as described  
35 (Brinster et al., Proc. Natl. Acad. Sci. USA 82, 4338  
(1985)), except that injection needles were beveled and



siliconized before use. Embryos were cultured overnight in a CO<sub>2</sub> incubator and 15 to 20 2-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice.

5           Following term pregnancy, 49 offspring were obtained from implantation of microinjected embryos. The offspring were screened by PCR amplification of the integrated transgene in genomic DNA samples. The target region for amplification was a 369 bp region of the  
10 human Apo E intron which was included in the expression vector. The oligos used for PCR amplification were:

5'- GCC TCT AGA AAG AGC TGG GAC-3' (SEQ ID NO:11)

5'- CGC CGT GTT CCA TTT ATG AGC-3' (SEQ ID NO:12)

15

          The conditions for PCR were: 94°C for 2 minute, 1 cycle; 94°C for 1 min, 63°C for 20 sec, and 72°C for 30 sec, 30 cycles. Of the 49 original offspring, 9 were identified as PCR positive transgenic  
20 founders.

          At 8-10 weeks of age, five transgenic founders (2, 11, 16, 17, and 28) and five controls ( 1, 12, 15, 18, and 30) were sacrificed for necropsy and pathological analysis. Liver was isolated from the  
25 remaining 4 founders by partial hepatectomy. For partial hepatectomy, the mice were anesthetized and a lobe of liver was surgically removed. Total cellular RNA was isolated from livers of all transgenic founders, and 5 negative control littermates as  
30 described (McDonald et al. Meth. Enzymol. 152, 219 (1987)). Northern blot analysis was performed on these samples to assess the level of transgene expression. Approximately 10ug of total RNA from each animal liver was resolved by electrophoresis denaturing gels (Ogden  
35 et al. Meth. Enzymol 152, 61 (1987)), then transferred to HYBOND-N nylon membrane (Amersham), and probed with

002215 09415725 11200

<sup>32</sup>P dCTP-labelled pB1.1 insert DNA. Hybridization was performed overnight at 42°C in 50% Formamide, 5 x SSPE, 0.5% SDS, 5 x Denhardt's solution, 100 µg/ml denatured salmon sperm DNA and 2-4 x 10<sup>6</sup> cpm of labeled probe/ml of hybridization buffer. Following hybridization, blots were washed twice in 2 x SSC, 0.1% SDS at room temperature for 5 min each, and then twice in 0.1 x SSC, 0.1% SDS at 55°C for 5-10 min each. Expression of the transgene in founder and control littermates was determined following autoradiography.

The northern blot data indicate that 7 of the transgenic founders express detectable levels of the transgene mRNA (animal #'s 2,11,16,17,22,33,and 45). The negative control mice and one of the founders (#28) expressed no transgene-related mRNA. Since OPG is predicted to be a secreted protein, overexpression of transgene mRNA should be a proxy for the level of systemically delivered gene product. Of the PCR and northern blot positive mice, animal 2, 17 and 22 expressed the highest levels of transgene mRNA, and may show more extensive biological effects on host cells and tissues.

25

#### EXAMPLE 4

##### Biological activity of OPG

Five of the transgenic mice (animals 2,11,16,17 and 28) and 5 control littermates (animals 1,12,15,18, and 30) were sacrificed for necropsy and pathological analysis using the following procedures: Prior to euthanasia, all animals had their identification numbers verified, then were weighed, anesthetized and blood drawn. The blood was saved as both serum and whole blood for a complete serum chemistry and hematology panel. Radiography was

performed just after terminal anesthesia by lethal CO<sub>2</sub> inhalation, and prior to the gross dissection.

Following this, tissues were removed and fixed in 10% buffered Zn-Formalin for histological examination. The

5 tissues collected included the liver, spleen, pancreas, stomach, duodenum, ileum, colon, kidney, reproductive organs, skin and mammary glands, bone, brain, heart, lung, thymus, trachea, esophagus, thyroid, jejunum, cecum, rectum, adrenals, urinary bladder, and skeletal  
10 muscle. Prior to fixation the whole organ weights were determined for the liver, stomach, kidney, adrenals, spleen, and thymus. After fixation the tissues were processed into paraffin blocks, and 3  $\mu$ m sections were obtained. Bone tissue was decalcified using a formic  
15 acid solution, and all sections were stained with hematoxylin and eosin. In addition, staining with Gomori's reticulin and Masson's trichrome were performed on certain tissues. Enzyme histochemistry was performed to determine the expression of tartrate  
20 resistant acid phosphatase (TRAP), an enzyme highly expressed by osteoclasts, multinucleated bone-resorbing cells of monocyte-macrophage lineage.

Immunohistochemistry for BrdU and F480 monocyte-macrophage surface antigen was also performed to detect  
25 replicating cells and cells of the monocyte-macrophage lineage, respectively. To detect F480 surface antigen expression, formalin fixed, paraffin embedded 4  $\mu$ m sections were deparaffinized and hydrated to deionized water. The sections were quenched with 3% hydrogen  
30 peroxide, blocked with Protein Block (Lipshaw, Pittsburgh, PA), and incubated in rat monoclonal anti-mouse F480 (Harlan, Indianapolis, IN). This antibody was detected by biotinylated rabbit anti-rat immunoglobulins, peroxidase conjugated streptavidin  
35 (BioGenex San Ramon, CA) with DAB as chromagen (BioTek,

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Santa Barbara, CA). Sections were counterstained with hematoxylin.

5 Upon gross dissection and observation of visceral tissues, no abnormalities were found in the transgene expressors or control littermates. Analysis of organ weight indicate that spleen size increased by approximately 38% in the transgenic mice relative to controls. There was a slight enlargement of platelet size and increased circulating unstained cells in the  
10 transgene expressors. There was a marginal decrease in platelet levels in the transgene expressors. In addition, the serum uric acid, urea nitrogen, and alkaline phosphatase levels all trended lower in the transgene expressors. The expressors were found to  
15 have increased radiodensity of the skeleton, including long bones (femurs), vertebrae, and flat bones (pelvis). The relative size of femurs in the expressors were not different from the the control mice.

20 Histological analysis of stained sections of bone from the OPG expressors show severe osteopetrosis with the presence of cartilage remnants from the primary spongiosa seen within bone trabeculae in the diaphysis of the femur. A clearly defined cortex was  
25 not identifiable in the sections of femur. In normal animals, the central diaphysis is filled with bone marrow. Sections of vertebra also show osteopetrotic changes implying that the OPG-induced skeletal changes were systemic. The residual bone marrow showed  
30 predominantly myeloid elements. Megakaryocytes were present. Reticulin stains showed no evidence for reticulin deposition. Immunohistochemistry for F480, a cell surface antigen expressed by cells of monocyte-macrophage derivation in the mouse, showed the presence  
35 of F480 positive cells in the marrow spaces. Focally,

flattened F480 positive cells could be seen directly adjacent to trabecular bone surfaces.

5 The mesenchymal cells lining the bony  
trabeculae were flattened and appeared inactive. Based  
on H&E and TRAP stains, osteoclasts were rarely found  
on the trabecular bone surfaces in the OPG expressors.  
In contrast, osteoclasts and/or chondroclasts were seen  
in the region of the growth plate resorbing cartilage,  
but their numbers may be reduced compared to controls.  
10 Also, osteoclasts were present on the cortical surface  
of the metaphysis where modelling activity is usually  
robust. The predominant difference between the  
expressors and controls was the profound decrease in  
trabecular osteoclasts, both in the vertebrae and  
15 femurs. The extent of bone accumulation was directly  
correlated with the level of OPG transgene mRNA  
detected by northern blotting of total liver RNA.

The spleens from the OPG expressors had an  
increased amount of red pulp with the expansion due to  
20 increased hematopoiesis. All hematopoietic lineages are  
represented. F480 positive cells were present in both  
control and OPG expressors in the red pulp. Two of the  
expressors (2 and 17) had foci of extramedullary  
hematopoiesis within the liver and this is likely due  
25 to the osteopetrotic marrow.

There were no observable abnormalities in  
the thymus, lymph nodes, gastrointestinal tract,  
pancreato-hepatobiliary tract, respiratory tract,  
reproductive system, genito-urinary system, skin,  
30 nervous system, heart and aorta, breast, skeletal  
muscle and fat.

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EXAMPLE 5

Isolation of mouse and human OPG cDNA

A cDNA clone corresponding to the 5' end of  
5 the mouse OPG mRNA was isolated from a mouse kidney  
cDNA library (Clontech) by PCR amplification. The  
oligonucleotides were derived from the rat OPG cDNA  
sequence and are shown below:

10 5'-ATCAAAGGCAGGGCATACTTCCTG-3' (SEQ ID NO:13)  
5'-GTTGCACTCCTGTTTCACGGTCTG-3' (SEQ ID NO:14)  
  
5'-CAAGACACCTTGAAGGGCCTGATG-3' (SEQ ID NO:15)  
5'-TAACTTTTACAGAAGAGCATCAGC-3' (SEQ ID NO:16)  
15  
5'-AGCGCGGCCGCATGAACAAGTGGCTGTGCTGCG-3' (SEQ ID NO:17)  
5'-AGCTCTAGAGAAACAGCCCAGTGACCATTCC-3' (SEQ ID NO:18)

The partial and full-length cDNA products  
20 obtained in this process were sequenced. The full-  
length product was digested with Not I and Xba I, then  
directionally cloned into the plasmid vector pRcCMV  
(Invitrogen). The resulting plasmid was named pRcCMV-  
Mu-OPG. The nucleotide sequence of the cloned product  
25 was compared to the rat OPG cDNA sequence. Over the  
1300 bp region spanning the OPG LORF, the rat and mouse  
DNA sequences are approximately 88% identical. The  
mouse cDNA sequence contained a 401 aa LORF, which was  
compared to the rat OPG protein sequence and found to  
30 be ~94% identical without gaps. This indicates that  
the mouse cDNA sequence isolated encodes the murine OPG  
protein, and that the sequence and structure has been  
highly conserved throughout evolution. The mouse OPG  
protein sequence contains an identical putative signal  
35 peptide at its N-terminus, and all 4 potential sites of  
N-linked glycosylation are conserved.

A partial human OPG cDNA was cloned from a human kidney cDNA library using the following rat-specific oligonucleotides:

- 5            5'-GTG AAG CTG TGC AAG AAC CTG ATG-3'  
            (SEQ ID NO:19)  
            5'-ATC AAA GGC AGG GCA TAC TTC CTG-3'  
            (SEQ ID NO:20)

- 10            This PCR product was sequenced and used to design primers for amplifying the 3' end of the human cDNA using a human OPG genomic clone in lambda as template:

- 15            5'-TCCGTAAGAAACAGCCCAGTGACC-3' (SEQ ID NO:29)  
            5'-CAGATCCTGAAGCTGCTCAGTTTG-3' (SEQ ID NO:21)

- The amplified PCR product was sequenced, and together with the 5' end sequence, was used to design  
20            5' and 3' human-specific primers useful for amplifying the entire human OPG cDNA coding sequences:

- 5'-AGCGCGGCCGCGGGGACCACAATGAACAAGTTG-3' (SEQ ID NO:22)  
            5'-AGCTCTAGAATTGTGAGGAAACAGCTCAATGGC-3' (SEQ ID NO:23)  
25

- The full-length human PCR product was sequenced, then directionally cloned into the plasmid vector pRcCMV (Invitrogen) using Not I and Xba I. The resulting plasmid was named pRcCMV-human OPG. The  
30            nucleotide sequence of the cloned product was compared to the rat and mouse OPG cDNA sequences. Over the 1300 bp region spanning the OPG LORF, the rat and mouse DNA sequences are approximately 78-88% identical to the human OPG cDNA. The human OPG cDNA sequence also  
35            contained a 401 aa LORF, and it was compared to the rat and mouse protein sequences. The predicted human OPG

protein is approximately 85% identical, and ~90% identical to the rat and mouse proteins, respectively. Sequence alignment of rat, mouse and human proteins show that they have been highly conserved during evolution. The human protein is predicted to have a N-terminal signal peptide, and 5 potential sites of N-linked glycosylation, 4 of which are conserved between the rat and mouse OPG proteins.

The DNA and predicted amino acid sequence of mouse OPG is shown in Figure 9A and 9B (SEQ ID NO:122). The DNA and predicted amino acid sequence of human OPG is shown in Figure 9C and 9D (SEQ ID NO:124). A comparison of the rat, mouse and human OPG amino acid sequences is shown in Figure 9E and 9F.

Isolation of additional human OPG cDNA clones revealed the presence of a G to C base change at position 103 of the DNA sequence shown in Figure 9C. This nucleotide change results in substitution of an asparagine for a lysine at position 3 of the amino acid sequence shown in Figure 9C. The remainder of the sequence in clones having this change was identical to that in Figure 9C and 9D.

25

#### EXAMPLE 6

#### OPG three-dimensional structure modelling

The amino-terminal portion of OPG has homology to the extracellular portion of all known members of the TNFR superfamily (Figure 1C). The most notable motif in this region of TNFR-related genes is an ~40 amino acid, cysteine-rich repeat sequence which folds into distinct structures (Banner et al. Cell 73, 431-445 (1993)). This motif is usually displayed in four (range 3-6) tandem repeats (see Figure 1C), and is known to be involved in ligand binding (Beutler and van



Huffel Science 264, 667-663 (1994)). Each repeat usually contains six interspaced cysteine residues, which are involved in forming three intradomain disulfide bonds, termed SS1, SS2, and SS3 (Banner et al., ibid). In some receptors, such as TNFR2, CD30 and CD40, some of the repeat domains contain only two intrachain disulfide bonds (SS1 and SS3).

The human OPG protein sequence was aligned to a TNFR1 extracellular domain profile using methods described by Luethy, et al., ibid, and the results were graphically displayed using the PrettyPlot program from the Wisconsin Package, version 8.1 (Genetics Computer Group, Madison, WI) (Figure 10). The alignment indicates a clear conservation of cysteine residues involved in formation of domains 1-4. This alignment was then used to construct a three-dimensional (3-D) model of the human OPG N-terminal domain using the known 3-D structure of the extracellular domain of p55 TNFR1 (Banner et al., ibid) as the template. To do this the atomic coordinates of the peptide backbone and side chains of identical residues were copied from the crystal structure coordinates of TNFR1. Following this, the remaining coordinates for the insertions and different side chains were generated using the LOOK program (Molecular Applications Group, Palo Alto, CA). The 3-D model was then refined by minimizing its conformational energy using LOOK.

By analogy with other TNFR family members, it is assumed that OPG binds to a ligand. For the purpose of modelling the interaction of OPG with its ligand, the crystal structure of TNF- $\beta$  was used to simulate a 3-D representation of an "OPG ligand". This data was graphically displayed (see Figure 11) using Molscript (Kraulis, J. Appl. Cryst. 24, 946-950, 1991). A model for the OPG/ligand complex with 3 TNF $\beta$  and 3 OPG molecules was constructed where the relative positions

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5

10

## 15

20

## Domain 2

35

contacts with TNF $\beta$  (see above), and may interact with  
an OPG ligand. In particular residues P66, H68, Y69,  
Y70, T71, D72, S73, H75, T76, S77, D78, E79, L81, Y82,  
P85, V86, K88, E89, L90, and Q91 are predicted to  
5 interact with a bound ligand based on our structural  
data.

#### Domain 3

Contains 4 cysteines involved in SS1 (C107 to  
10 C 118) and SS3 (C124 to C142) disulfide bonds, but not  
an SS2 bond. Based on our structural data, residues  
E115, L118 and K119 are predicted in to interact with  
an OPG ligand.

#### 15 Domain 4

Contains 4 cysteines involved in SS1 (C145 to  
C160) and SS3 (C166 to C185) disulfide bonds, but not  
an SS2 bond, similar to domain 3. Our structural data  
predict that E153 and S155 interact with an OPG ligand.

20 Thus, the predicted structural model for OPG  
identifies a number of highly conserved residues which  
are likely to be important for its biological activity.

25

#### EXAMPLE 7

##### Production of recombinant secreted OPG protein in mammalian cells

30 To determine if OPG is actually a secreted  
protein, mouse OPG cDNA was fused to the human IgG1 Fc  
domain as a tag (Capon et al. Nature 337, 525-531  
(1989)), and expressed in human 293 fibroblasts. Fc  
fusions were carried out using the vector pFc-A3. pFc-  
35 A3 contains the region encoding the Fc portion of human

immunoglobulin IgG- $\gamma$ 1 heavy chain (Ellison et al. *ibid*)  
from the first amino acid of the hinge domain (Glu-99)  
to the carboxyl terminus and is flanked by a 5'-NotI  
fusion site and 3'-SalI and XbaI sites. The plasmid  
5 was constructed by PCR amplification of the human  
spleen cDNA library (Clontech). PCR reactions were in a  
final volume of 100  $\mu$ l and employed 2 units of Vent DNA  
polymerase (New England Biolabs) in 20 mM Tris-HCl (pH  
8.8), 10 mM KCl, 10  $\mu$ M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.1%  
10 Triton X-100 with 400  $\mu$ M each dNTP and 1 ng of the cDNA  
library to be amplified together with 1  $\mu$ M of each  
primer. Reactions were initiated by denaturation at  
95°C for 2 min, followed by 30 cycles of 95°C for 30 s,  
55°C for 30 s, and 73°C for 2 min. The 5' primer  
15 5' ATAGCGGCCGCTGAGCCCAAATCTTGTGACAAACTCAC 3' (SEQ  
ID NO:24)  
incorporated a NotI site immediately 5' to the first  
residue (Glu-99) of the hinge domain of IgG- $\gamma$ 1. The 3'  
primer  
20 5'-TCTAGAGTCGACTTATCATTTACCCGGAGACAGGGAGAGGCTCTT-  
3' (SEQ ID NO:25)  
incorporated SalI and XbaI sites. The 717-bp PCR  
product was digested with NotI and SalI, isolated by  
electrophoresis through 1% agarose (FMC Corp.), purified  
25 by the GeneClean procedure (BIO 101, Inc.) and cloned  
into NotI, SalI-digested pBluescript II KS vector  
(Stratagene). The insert in the resulting plasmid, pFc-  
A3, was sequenced to confirm the fidelity of the PCR  
reaction.  
30 The cloned mouse cDNA in plasmid pRcCMV-MuOPG  
was amplified using the following two sets of primer  
pairs:

Pair 1

09713786 14200

5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAG-3' (SEQ ID NO:26)

5'-CCTCTGCGGCCGCTAAGCAGCTTATTTTCACGGATTGAACCTG-3' (SEQ ID NO:27)

5

Pair 2

5'-CCTCTGAGCTCAAGCTTCCGAGGACCACAATGAACAAG-3' (SEQ ID NO:28)

10 5'-CCTCTGCGGCCGCTGTTGCATTTCTTTCTG-3' (SEQ ID NO:30)

09748725 19900

The first pair amplifies the entire OPG LORF, and creates a NotI restriction site which is compatible with the in-frame Not I site in Fc fusion vector pFcA3. pFcA3 was prepared by engineering a NotI restriction site 5' to aspartic acid residue 216 of the human IgG1 Fc cDNA. This construct introduces a linker which encodes two irrelevant amino acids which span the junction between the OPG protein and the IgG Fc region. This product, when linked to the Fc portion, would encode all 401 OPG residues directly followed by all 227 amino acid residues of the human IgG1 Fc region (Fl.Fc). The second primer pair amplifies the DNA sequences encoding the first 180 amino acid residues of OPG, which encompasses its putative ligand binding domain. As above, the 3' primer creates an artificial Not I restriction site which fuses the C-terminal truncated OPG LORF at position threonine 180 directly to the IgG1 Fc domain (CT.fc).

30 The amino acid sequence junction linking OPG residue 401 and aspartic acid residue 221 of the human Fc region can be modified as follows: The DNA encoding residues 216-220 of the human Fc region can be deleted as described below, or the cysteine residue  
35 corresponding to C220 of the human Fc region can be mutated to either serine or alanine. OPF-Fc fusion

protein encoded by these modified vectors can be transfected into human 293 cells, or CHO cells, and recombinant OPG-Fc fusion protein purified as described below.

5 Both products were directionally cloned into the plasmid vector pCEP4 (Invitrogen). pCEP4 contains the Epstein-Barr virus origin of replication, and is capable of episomal replication in 293-EBNA-1 cells. The parent pCEP4, and pCEP4-F1.Fc and pCEP4-CT.Fc  
10 vectors were lipofected into 293-EBNA-1 cells using the manufacturer's recommended methods. The transfected cells were then selected in 100 µg/ml hygromycin to select for vector expression, and the resulting drug-resistant mass cultures were grown to confluence. The  
15 cells were then cultured in serum-free media for 72 hr, and the conditioned media removed and analysed by SDS-PAGE. A silver staining of the polyacrylamide gel detects the major conditioned media proteins produced by the drug resistant 293 cultures. In the pCEP4-F1.Fc  
20 and the pCEP4-CT.Fc conditioned media, unique bands of the predicted sizes were abundantly secreted (see Figures 13B and 13C). The full-length Fc fusion protein accumulated to a high concentration, indicating that it may be stable. Both Fc fusion proteins were  
25 detected by anti-human IgG1 Fc antibodies (Pierce) on western blots, indicating that they are recombinant OPG products.

The full length OPG-Fc fusion protein was purified by Protein-A column chromatography (Pierce)  
30 using the manufacturers recommended procedures. The protein was then subjected to N-terminal sequence analysis by automated Edman degradation as essentially described by Matsudaira et al. (J. Biol. Chem. 262, 10-35 (1987)). The following amino acid sequence was read  
35 after 19 cycles:

NH2-E T L P P K Y L H Y D P E T G H Q L L-CO2H  
(SEQ ID NO:31)

5           This sequence was identical to the predicted  
mouse OPG amino acid sequence beginning at amino acid  
residue 22, suggesting that the natural mammalian  
leader cleavage site is between amino acid residues  
Q21-E22, not between Y31-D32 as originally predicted.  
10 The expression experiments performed in 293-EBNA cells  
with pCEP4-F1.Fc and pCEP4-CT.Fc demonstrate that OPG  
is a secreted protein, and may act systemically to bind  
its ligand.

          Procedures similar to those used to construct  
15 and express the muOPG[22-180]-Fc and muOPG[22-401]-Fc  
fusions were employed for additional mouse and human  
OPG-Fc fusion proteins.

          Murine OPG cDNA encoding amino acids 1-185  
fused to the Fc region of human IgG1 [muOPG Ct(185).Fc]  
20 was constructed as follows. Murine OPG cDNA from  
plasmid pRcCMV Mu Osteoprotegerin (described in Example  
5) was amplified using the following primer pair in a  
polymerase chain reaction as described above:

25 1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:32)

1333-80:

5'-CCT CTG CGG CCG CAC ACA CGT TGT CAT GTG TTG C-  
3' (SEQ ID NO:33)

30

          This primer pair amplifies the murine OPG  
cDNA region encoding amino acid residues 63-185  
(corresponding to bp 278-645) of the OPG reading frame  
as shown in Figure 9A. The 3' primer contains a Not I  
35 restriction site which is compatible with the in-frame  
Not I site of the Fc fusion vector pFcA3. The product

094336-1000

also spans a unique EcoRI restriction site located at  
bp 436. The amplified PCR product was purified,  
cleaved with NotI and EcoRI, and the resulting EcoRI-  
NotI restriction fragment was purified. The vector  
5 pCEP4 having the murine 1-401 OPG-Fc fusion insert was  
cleaved with EcoRI and NotI, purified, and ligated to  
the PCR product generated above. The resulting pCEP4-  
based expression vector encodes OPG residues 1-185  
10 directly followed by all 227 amino acid residues of the  
human IgG1 Fc region. The murine OPG 1-185.Fc fusion  
vector was transfected into 293 cells, drug selected,  
and conditioned media was produced as described above.  
The resulting secreted murine OPG 1-185.Fc fusion  
15 product was purified by Protein-A column chromatography  
(Pierce) using the manufacturers recommended  
procedures.

Murine OPG DNA encoding amino acid residues  
1-194 fused to the Fc region of human IgG1 (muOPG  
20 Ct(194).Fc) was constructed as follows. Mouse OPG cDNA  
from plasmid pRcCMV Mu-Osteoprotegerin was amplified  
using the following primer pairs:

1333-82:

25 5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:34)

1333-81:

5'-CCT CTG CGG CCG CCT TTT GCG TGG CTT CTC TGT T-  
3' (SEQ ID NO:35)

30 This primer pair amplifies the murine OPG  
cDNA region encoding amino acid residues 70-194  
(corresponding to bp 298-672) of the OPG reading frame.  
The 3' primer contains a Not I restriction site which  
is compatible with the in-frame Not I site of the Fc  
35 fusion vector pFcA3. The product also spans a unique  
EcoRI restriction site located at bp 436. The



amplified PCR product was cloned into the murine OPG[1-401] Fc fusion vector as described above. The resulting pCEP4-based expression vector encodes OPG residues 1-194 directly followed by all 227 amino acid residues of the human IgG1 Fc region. The murine OPG 1-194.Fc fusion vector was transfected into 293 cells, drug selected, and conditioned media was produced. The resulting secreted fusion product was purified by Protein-A column chromatography (Pierce) using the manufacturers recommended procedures.

Human OPG DNA encoding amino acids 1-401 fused to the Fc region of human IgG1 was constructed as follows. Human OPG DNA in plasmid pRcCMV-hu osteoprotegerin (described in Example 5) was amplified using the following oligonucleotide primers:

1254-90:

5'CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT G-3'  
(SEQ ID NO:36)

1254-95:

5'-CCT CTG CGG CCG CTA AGC AGC TTA TTT TTA CTG AAT GG-3'  
(SEQ ID NO:37)

25

The resulting PCR product encodes the full-length human OPG protein and creates a Not I restriction site which is compatible with the in-frame Not I site Fc fusion vector FcA3. The PCR product was directionally cloned into the plasmid vector pCEP4 as described above. The resulting expression vector encodes human OPG residues 1-401 directly followed by 227 amino acid residues of the human IgG1 Fc region. Conditioned media from transfected and drug selected cells was produced and the huOPG F1.Fc fusion product was purified by Protein-A column chromatography

(Pierce) using the manufacturers recommended procedures.

Human OPG DNA encoding amino acid residues 1-  
5 201 fused to the Fc region of human IgG1 [huOPG  
Ct(201).Fc] was constructed as follows. The cloned  
human OPG cDNA from plasmid pRrCMV-hu osteoprotegerin  
was amplified by PCR using the following  
oligonucleotide primer pair:

10

1254-90:

5'-CCT CTG AGC TCA AGC TTG GTT TCC GGG GAC CAC AAT  
G-3' (SEQ ID NO:38)

1254-92:

15

5'-CCT CTG CGG CCG CCA GGG TAA CAT CTA TTC CAC-3'  
(SEQ ID NO:39)

This primer pair amplifies the human OPG cDNA  
region encoding amino acid residues 1-201 of the OPG  
20 reading frame, and creates a Not I restriction site at  
the 3' end which is compatible with the in-frame Not I  
site Fc fusion vector FcA3. This product, when linked  
to the Fc portion, encodes OPG residues 1-201 directly  
followed by all 221 amino acid residues of the human  
25 IgG1 Fc region. The PCR product was directionally  
cloned into the plasmid vector pCEP4 as described  
above. Conditioned media from transfected and drug  
selected cells was produced, and the hu OPG Ct(201).Fc  
fusion products purified by Protein-A column  
30 chromatography (Pierce) using the manufacturer's  
recommended procedures.

The following procedures were used to  
construct and express unfused mouse and human OPG.

35

A plasmid for mammalian expression of full-  
length murine OPG (residues 1-401) was generated by PCR

amplification of the murine OPG cDNA insert from pRcCMV  
Mu-Osteoprotegerin and subcloned into the expression  
vector pDSR $\alpha$  (DeClerck et. al. J. Biol. Chem. 266,  
3893 (1991)). The following oligonucleotide primers  
5 were used:

1295-26:

5'-CCG AAG CTT CCA CCA TGA ACA AGT GGC TGT GCT  
GC-3' (SEQ ID NO:40)

10

1295-27:

5'-CCT CTG TCG ACT ATT ATA AGC AGC TTA TTT TCA CGG  
ATT G-3' (SEQ ID NO:41)

15

The murine OPG full length reading frame was  
amplified by PCR as described above. The PCR product  
was purified and digested with restriction  
endonucleases Hind III and Xba I (Boehringer Mannheim,  
Indianapolis, IN) under the manufacturers recommended  
20 conditions, then ligated to Hind III and Xba I digested  
pDSR $\alpha$ . Recombinant clones were detected by restriction  
endonuclease digestion, then sequenced to ensure no  
mutations were produced during the PCR amplification  
steps.

25

The resulting plasmid, pDSR $\alpha$ -muOPG was  
introduced into Chinese hamster ovary (CHO) cells by  
calcium mediated transfection (Wigler et al. Cell 11,  
233 (1977)). Individual colonies were selected based  
upon expression of the dihydrofolate reductase (DHFR)  
30 gene in the plasmid vector and several clones were  
isolated. Expression of the murine OPG recombinant  
protein was monitored by western blot analysis of CHO  
cell conditioned media. High expressing cells were  
selected, and OPG expression was further amplified by  
35 treatment with methotrexate as described (DeClerck et  
al., idid). Conditioned media from CHO cell lines was

produced for further purification of recombinant secreted murine OPG protein.

5 A plasmid for mammalian expression of full-length human OPG (amino acids 1-401) was generated by subcloning the cDNA insert in pRcCMV-hu Osteoprotegerin directly into vector pDSR $\alpha$  (DeClerck et al., ibid). The pRcCMV-OPG plasmid was digested to completion with Not I, blunt ended with Klenow, then digested to completion  
10 with Xba I. Vector DNA was digested with Hind III, blunt ended with Klenow, then digested with Xba I, then ligated to the OPG insert. Recombinant plasmids were then sequenced to confirm proper orientation of the human OPG cDNA.

15 The resulting plasmid pDSR $\alpha$ -huOPG was introduced into Chinese hamster ovary (CHO) cells as described above. Individual colonies were selected based upon expression of the dihydrofolate reductase (DHFR) gene in the plasmid vector and several clones  
20 were isolated. Expression of the human OPG recombinant protein was monitored by western blot analysis of CHO cell conditioned media. High expressing clones were selected, and OPG expression was further amplified by treatment with methotrexate. Conditioned media from  
25 CHO cell lines expressing human OPG was produced for protein purification.

Expression vectors for murine OPG encoding residues 1-185 were constructed as follows. Murine OPG  
30 cDNA from pRcCMV-Mu OPG was amplified using the following oligonucleotide primers:

1333-82:

5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:42)

35 1356-12:

5'-CCT CTG TCG ACT TAA CAC ACG TTG TCA TGT GTT  
GC-3' (SEQ ID NO:43)

5 This primer pair amplifies the murine OPG  
cDNA region encoding amino acids 63-185 of the OPG  
reading frame (bp 278-645) and contains an artificial  
stop codon directly after the cysteine codon (C185),  
which is followed by an artificial Sal I restriction  
endonuclease site. The predicted product contains an  
10 internal Eco RI restriction site useful for subcloning  
into a pre-existing vector. After PCR amplification,  
the resulting purified product was cleaved with Eco RI  
and Sal I restriction endonucleases, and the large  
fragment was gel purified. The purified product was  
15 then subcloned into the large restriction fragment of  
an Eco RI and Sal I digest of pBluescript-muOPG Fl.Fc  
described above. The resulting plasmid was digested  
with Hind III and Xho I and the small fragment was gel  
purified. This fragment, which contains a open reading  
20 frame encoding residues 1-185 was then subcloned into a  
Hind III and Xho I digest of the expression vector  
pCEP4. The resulting vector, pmuOPG [1-185], encodes a  
truncated OPG polypeptide which terminates at a  
cysteine residue located at position 185. Conditioned  
25 media from transfected and drug selected cells was  
produced as described above.

1333-82:  
30 5'-TCC CTT GCC CTG ACC ACT CTT-3' (SEQ ID NO:44)

1356-13:  
5'-CCT CTG TCG ACT TAC TTT TGC GTG GCT TCT CTG  
TT-3' (SEQ ID NO:45)

35

This primer pair amplifies the murine OPG cDNA region encoding amino acids 70-194 of the OPG reading frame (bp 298-672) and contains an artificial stop codon directly after the lysine codon (K194), which is followed by an artificial Sal I restriction endonuclease site. The predicted product contains an internal Eco RI restriction site useful for subcloning into a pre-existing vector. After PCR amplification, the resulting purified product was cleaved with Eco RI and Sal I restriction endonucleases, and the large fragment was gel purified. The purified product was then subcloned into the large restriction fragment of an Eco RI and Sal I digest of pBluescript-muOPG F1.Fc described above. The resulting plasmid was digested with Hind III and Xho I and the small fragment was gel purified. This fragment, which contains a open reading frame encoding residues 1-185 was then subcloned into a Hind III and Xho I digest of the expression vector pCEP4. The resulting vector, pmuOPG [1-185], encodes a truncated OPG polypeptide which terminates at a lysine at position 194. Conditioned media from transfected and drug selected cells was produced as described above.

Several mutations were generated at the 5' end of the huOPG [22-401]-Fc gene that introduce either amino acid substitutions, or deletions, of OPG between residues 22 through 32. All mutations were generated with the "QuickChange™ Site-Directed Mutagenesis Kit" (Stratagene, San Diego, CA) using the manufacturer's recommended conditions. Briefly, reaction mix containing huOPG [22-401]-Fc plasmid DNA template and mutagenic primers were treated with Pfu polymerase in the presence of deoxynucleotides, then amplified in a thermocycler as described above. An aliquot of the reaction is then transfected into competent E. coli

XL1-Blue by heatshock, then plated. Plasmid DNA from transformants was then sequenced to verify mutations.

The following primer pairs were used to delete residues 22-26 of the human OPG gene, resulting in the production of a huOPG [27-401]-Fc fusion protein:

1436-11:

5'-TGG ACC ACC CAG AAG TAC CTT CAT TAT GAC-3'  
10 (SEQ ID NO:140)

1436-12:

5'-GTC ATA ATG AAG GTA CTT CTG GGT GGT CCA-3'  
15 (SEQ ID NO:141)

The following primer pairs were used to delete residues 22-28 of the human OPG gene, resulting in the production of a huOPG [29-401]-Fc fusion protein:

20

1436-17:

5'-GGA CCA CCC AGC TTC ATT ATG ACG AAG AAA C-3'  
(SEQ ID NO:142)

25 1436-18:

5'-GTT TCT TCG TCA TAA TGA AGC TGG GTG GTC C-3'  
(SEQ ID NO:143)

The following primer pairs were used to delete residues 22-31 of the human OPG gene, resulting in the production of a huOPG [32-401]-Fc fusion protein:

30

1436-27:

5'-GTG GAC CAC CCA GGA CGA AGA AAC CTC TC-3'  
(SEQ ID NO:144)

5 1436-28:

5'-GAG AGG TTT CTT CGT CCT GGG TGG TCC AC-3'  
(SEQ ID NO:145)

10 The following primer pairs were used to  
change the codon for tyrosine residue 28 to  
phenylalanine of the human OPG gene, resulting in the  
production of a huOPG [22-401]-Fc Y28F fusion protein:

1436-29:

15 5'-CGT TTC CTC CAA AGT TCC TTC ATT ATG AC-3'  
(SEQ ID NO:146)

1436-30:

20 5'-GTC ATA ATG AAG GAA CTT TGG AGG AAA CG-3'  
(SEQ ID NO:147)

The following primer pairs were used to  
change the codon for proline residue 26 to alanine of  
the human OPG gene, resulting in the production of a  
25 huOPG [22-401]-Fc P26A fusion protein:

1429-83:

5'-GGA AAC GTT TCC TGC AAA GTA CCT TCA TTA TG-3  
(SEQ ID NO:148)

30

1429-84:

5'-CAT AAT GAA GGT ACT TTG CAG GAA ACG TTT CC-3'  
(SEQ ID NO:149)

35

Each resulting muOPG [22-401]-Fc plasmid  
containing the appropriate mutation was then

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transfected into human 293 cells, the mutant OPG-Fc fusion protein purified from conditioned media as described above. The biological activity of each protein was assessed the in vitro osteoclast forming assay described in Example 11.

## EXAMPLE 8

### Expression of OPG in E. coli

10

#### A. Bacterial Expression Vectors

##### pAMG21

The expression plasmid pAMG21 can be derived from the Amgen expression vector pCFM1656 (ATCC #69576) which in turn be derived from the Amgen expression vector system described in US Patent No. 4,710,473. The pCFM1656 plasmid can be derived from the described pCFM836 plasmid (Patent No. 4,710,473) by: (a) destroying the two endogenous NdeI restriction sites by end filling with T4 polymerase enzyme followed by blunt end ligation; (b) replacing the DNA sequence between the unique AatII and ClaI restriction sites containing the synthetic P<sub>L</sub> promoter with a similar fragment obtained from pCFM636 (patent No. 4,710,473) containing the PL promoter

30     AatII  
5'     CTAATTCCGCTCTCACCTACCAAACAATGCCCCCTGCAAAAAATAAATTCATAT-  
3'     TGCAGATTAAGGCGAGAGTGGATGGTTTGTACGGGGGACGTTTTTTATTTAAGTATA-

-AAAAACATACAGATAACCATCTGCGGTGATAAATTATCTCTGGCGGTGTTGACATAAA-  
-TTTTTTGTATGTCTATTGGTAGACGCCACTATTTAATAGAGACCGCCACAACGTATTT-

35

-TACCACTGGCGGTGATACTGAGCACAT 3' (SEQ ID NO:53)  
-ATGGTGACCGCCACTATGACTCGTGTAGC5' (SEQ ID NO:54)

ClaI

and then (c) substituting the small DNA sequence between the unique *ClaI* and *KpnI* restriction sites with the following oligonucleotide:

5

5' CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC3'  
(SEQ ID NO:48)

3' TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGC 5'  
10 (SEQ ID NO:49)

*ClaI*

*KpnI*

The expression plasmid pAMG21 can then be derived from pCFM1656 by making a series of site directed base  
15 changes by PCR overlapping oligo mutagenesis and DNA sequence substitutions. Starting with the *BglII* site (plasmid bp # 180) immediately 5' to the plasmid replication promoter *PcopB* and proceeding toward the plasmid replication genes, the base pair changes are as  
20 follows:

	<u>pAMG21 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG21</u>
	# 204	T/A	C/G
25	# 428	A/T	G/C
	# 509	G/C	A/T
	# 617	- -	insert two G/C bp
	# 679	G/C	T/A
	# 980	T/A	C/G
30	# 994	G/C	A/T
	# 1004	A/T	C/G
	# 1007	C/G	T/A
	# 1028	A/T	T/A
	# 1047	C/G	T/A
35	# 1178	G/C	T/A
	# 1466	G/C	T/A
	# 2028	G/C	bp deletion
	# 2187	C/G	T/A
	# 2480	A/T	T/A
40	# 2499-2502	AGTG TCAC	GTCA CAGT
	# 2642	TCCGAGC AGGCTCG	7 bp deletion
45	# 3435	G/C	A/T
	# 3446	G/C	A/T
	# 3643	A/T	T/A

The DNA sequence between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites is substituted with the following DNA sequence:

[AatII sticky end] 5' GCGTAACGTATGCATGGTCTCC-  
10 (position #4358 in pAMG21) 3' TGCACGCATTGCATACGTACCAGAGG-  
-CCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACT-  
-GGTACGCTCTCATCCCTTGACGGTCCGTAGTTTATTTTGCTTTCCGAGTCAGCTTTTCTGA-  
15 -GGGCCTTTTCGTTTTATCTGTTGTTTGTGCGGTGAACGCTCTCCTGAGTAGGACAAATCCGC-  
-CCCGGAAAGCAAAATAGACAACAAACAGCCACTTGCGAGAGGACTCATCCTGTTTAGGCG-  
-CGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGCGGGCAGGACGCCCCG-  
20 -GCCCTCGCCTAAACTTGCAACGCTTCGTTGCCGGGCCTCCCACGCCCCGTCTGCGGGCG-  
-CATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGT-  
-GTATTTGACGGTCCGTAGTTTAATTCGTCTTCCGGTAGGACTGCCTACCGGAAAAACGCA-  
AatII  
25 -TTCTACAACTCTTTTGTATTATTTTCTAAATACATTCAAATATGGACGTCGTACTTAAC-  
-AAGATGTTTGAGAAAACAAATAAAAAGATTTATGTAAGTTTATACCTGCAGCATGAATTG-  
-TTTTAAAGTATGGGCAATCAATTGCTCCTGTAAAATTGCTTTAGAAATACTTTGGCAGC-  
30 -AAAATTTTCATACCCGTTAGTTAACGAGGACAATTTAACGAAATCTTTATGAAACCGTCG-  
-GGTTTGTGTTGATTGAGTTTCATTTGCGCATTTGGTTAAATGGAAAGTGACCGTGCGCTTAC-  
-CCAAACAACATAACTCAAAGTAAACGCGTAACCAATTTACCTTTCACTGGCACGCGAATG-  
-TACAGCCTAATATTTTTGAAATATCCCAAGAGCTTTTTCTTTCGTCATGCCCACGCTAAAC-  
35 -ATGTCGGATTATAAAAACTTTATAGGGTCTCGAAAAAGGAAGCGTACGGGTGCGATTTG-  
-ATTCTTTTTCTCTTTTGGTTAAATCGTTGTTTGATTTATTATTTGCTATATTTATTTTTC-  
-TAAGAAAAAGAGAAAACCAATTTAGCAACAACTAAATAATAAACGATATAAATAAAAAAG-  
40 -GATAATTATCAACTAGAGAAGGAACAATTAATGGTATGTTTCATACACGCATGTAAAAATA-  
-CTATTAATAGTTGATCTCTTCCTTGTTAATTACCATACAAGTATGTGCGTACATTTTAT-  
-AACTATCTATATAGTTGTCTTTCTCTGAATGTGCAAACTAAGCATTCCGAAGCCATTAT-  
45 -TTGATAGATATATCAACAGAAAGAGACTTACACGTTTTGATTTCGTAAGGCTTCGGTAATA-  
-TAGCAGTATGAATAGGGAACTAAACCCAGTGATAAGACCTGATGATTTTCGCTTCTTTAA-  
-ATCGTCATACTTATCCCTTTGATTTGGGTCATTCTTGGACTACTAAAGCGAAGAAATT-  
-TTACATTTGGAGATTTTTTATTTACAGCATTGTTTTCAAATATATTCCAATTAATCGGTG-  
50 -AATGTAAACCTCTAAAAAATAAATGTCGTAACAAAAGTTTATATAAGGTTAATTAGCCAC-  
-AATGATTGGAGTTAGAATAATCTACTATAGGATCATATTTTATTAAATTAGCGTCATCAT-  
-TTACTAACCTCAATCTTATTAGATGATATCCTAGTATAAATAATTTAATCGCAGTAGTA-  
55 -AATATTGCCTCCATTTTTTAGGGTAATTATCCAGAATTGAAATATCAGATTTAACCATAG-  
-TTATAACGGAGGTAAAAAATCCCATTAATAGGTCTTAACCTTTATAGTCTAAATTGGTATC-  
-AATGAGGATAAATGATCGCGAGTAAATAATATTCACAATGTACCATTTTAGTCATATCAG-

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5

10

15

20

SacII

25

30

-AACCGCTCTTCACGCTCTTCACGC 3' [SacII sticky end] (SEQ ID NO:50)  
-TTGGCGAGAAGTGCAGAAGTG 5' (position #5904 in pAMG21) (SEQ ID NO:46)

35 During the ligation of the sticky ends of this  
substitution DNA sequence, the outside AatII and SacII  
sites are destroyed. There are unique AatII and SacII  
sites in the substituted DNA.

40 pAMG22-His

The expression plasmid pAMG22-His can be derived from the Amgen expression vector pAMG22 by substituting the small DNA sequence between the unique NdeI ( #4795) and EcoRI ( #4818) restriction sites of pAMG22 with the following oligonucleotide duplex:

NdeI NheI EcoRI  
 5' TATGAAACATCATCACCATCACCATCATGCTAGCGTTAACGCGTTGG 3'  
 (SEQ ID NO:51)

50 3' ACTTTGTAGTAGTGGTAGTGGTAGTACGATCGCAATTGCGCAACCTTAA 5'  
(SEQ ID NO:52)

MetLysHisHisHisHisHisHisHisAlaSerValAsnAlaLeuGlu  
(SEQ ID NO:168)

pAMG22

5 The expression plasmid pAMG22 can be derived from the  
Amgen expression vector pCFM1656 (ATCC #69576) which in  
turn be derived from the Amgen expression vector system  
described in US Patent No. 4,710,473 granted December  
1, 1987. The pCFM1656 plasmid can be derived from the  
10 described pCFM836 plasmid (Patent No. 4,710,473) by:  
(a) destroying the two endogenous NdeI restriction  
sites by end filling with T4 polymerase enzyme followed  
by blunt end ligation; (b) replacing the DNA sequence  
between the unique AatII and ClaI restriction sites  
15 containing the synthetic PL promoter with a similar  
fragment obtained from pCFM636 (patent No. 4,710,473)  
containing the PL promoter

AatII

20 5' CTAATTCGCTCTCACCTACCAAACAATGCCCCCTGCAAAAAATAAATTCATAT-  
3'TGCAGATTAAGGCGAGAGTGGATGGTTTGTACGGGGGACGTTTTTTATTTAAGTATA-  
  
-AAAAAACATACAGATAACCATCTGCGGTGATAAATTATCTCTGGCGGTGTTGACATAAA-  
-TTTTTTGTATGTCTATTGGTAGACGCCACTATTTAATAGAGACCGCCACAACGTATTT-  
25  
-TACCACTGGCGGTGATACTGAGCACAT 3' (SEQ ID NO:53)  
-ATGGTGACCGCCACTATGACTCGTGTAGC5' (SEQ ID NO:54)

ClaI

30 and then (c) substituting the small DNA sequence  
between the unique *ClaI* and *KpnI* restriction sites  
with the following oligonucleotide:

5' CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC 3'  
35 (SEQ ID NO:55)

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3' TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGC 5'  
(SEQ ID NO:56)

ClaI

KpnI

5 The expression plasmid pAMG22 can then be derived from  
pCFM1656 by making a series of site directed base  
changes by PCR overlapping oligo mutagenesis and DNA  
sequence substitutions. Starting with the BglIII site  
(plasmid bp # 180) immediately 5' to the plasmid  
10 replication promoter PcopB and proceeding toward the  
plasmid replication genes, the base pair changes are as  
follows:

	<u>pAMG22 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG22</u>
15	# 204	T/A	C/G
	# 428	A/T	G/C
20	# 509	G/C	A/T
	# 617	- -	insert two G/C bp
	# 679	G/C	T/A
	# 980	T/A	C/G
25	# 994	G/C	A/T
	# 1004	A/T	C/G
	# 1007	C/G	T/A
	# 1028	A/T	T/A
	# 1047	C/G	T/A
30	# 1178	G/C	T/A
	# 1466	G/C	T/A
	# 2028	G/C	bp deletion
	# 2187	C/G	T/A
	# 2480	A/T	T/A
35	# 2499-2502	AGTG TCAC	GTCA CAGT
	# 2642	TCCGAGC AGGCTCG	7 bp deletion
40	# 3435	G/C	A/T
	# 3446	G/C	A/T
	# 3643	A/T	T/A
45			

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The DNA sequence between the unique AatII (position #4364 in pCFM1656) and SacII (position #4585 in pCFM1656) restriction sites is substituted with the following DNA sequence:

5

[AatII sticky end] (position #4358 in pAMG22)

```

5'      GCGTAACGTATGCATGGTCTCCCCATGCGAGAGTAGGGAAGTCCAGGCATCAA-
3' TGCACGCATTGCATACGTACCAGAGGGGTACGCTCTCATCCCTTGACGGTCCGTAGTT-
10 -ATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCTGGTG-
   -TATTTTGCTTTCCGAGTCAGCTTCTGACCCGGAAGCAAATAGACAACAAACAGCCAC-

15 -AACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGG-
   -TTGCGAGAGGACTCATCTGTTTAGCGGGCCCTCGCCTAAACTTGCAACGCTTCGTTGCC-

   -CCCGGAGGGTGGCGGGCAGGACGCCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAG-
20 -GGGCCTCCCAACGCCCGTCTGCGGGCGGTATTTGACGGTCCGTAGTTTAATTCGTCTTC-

   -GCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAACTCTTTTGTATTATTTTCTAAAT-
   -CGGTAGGACTGCCTACCGGAAAAACGCAAAGATGTTTGAGAAAACAAATAAAAAGATTTA-

                        AatII
25 -ACATTCAAATATGGACGTCTCATAATTTTTAAAAAATTCATTTGACAAATGCTAAAATTC-
   -TGTAAGTTTATACCTGCAGAGTATTAAAAATTTTTTAAGTAAACTGTTTACGATTTTAAG-

   -TTGATTAATATTTCTCAATTGTGAGCGCTCACAATTTATCGATTTGATTCTAGATTTGTTT-
30 -AACTAATTATAAGAGTTAACACTCGCGAGTGTTAAATAGCTAAACTAAGATCTAAACTCA-

   -TAACTAATTAAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGAGCTCACTAGTGT-
   -ATTGATTAATTTCTCCTTATTGTATACCAATTGCGCAACCTTAAGCTCGAGTGATCACA-

                        SacII
35 -CGACCTGCAGGGTACCATGGAAGCTTACTCGAGGATCCGCGGAAGAAGAAGAAGAGAA-
   -GCTGGACGTCCCATGGTACCTTCGAATGAGCTCCTAGGCGCCTTCTTCTTCTTCTT-

   -GAAAGCCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACC-
40 -CTTTCGGGCTTTCCTTCGACTCAACCGACGACGGTGGCGACTCGTTATTGATCGTATTGG-

   -CCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACCGCTCTTCA-
   -GGAACCCCGGAGATTTGCCCAAGAACTCCCCAAAAACGACTTTCCTCCTTGCGGAGAAGT-

   -CGCTCTTACGC 3' (SEQ ID NO:58)
45 -GCGAGAAGTG 5' (SEQ ID NO:57)

```

[SacII sticky end] (position #5024 in pAMG22)

During the ligation of the sticky ends of this substitution DNA sequence, the outside AatII and SacII sites are destroyed. There are unique AatII and SacII sites in the substituted DNA.

B. Human OPG Met[32-401]

In the example, the expression vector used was pAMG21, a derivative of pCFM1656 (ATCC accession no. 69576) which contains appropriate restriction sites for insertion of genes downstream from the lux PR promoter. (See U.S. Patent No. 5,169,318 for description of the lux expression system). The host cell used was GM120 (ATCC accession no. 55764). This host has the lacIQ promoter and lacI gene integrated into a second site in the host chromosome of a prototrophic E. coli K12 host. Other commonly used E. coli expression vectors and host cells are also suitable for expression.

A DNA sequence coding for an N-terminal methionine and amino acids 32-401 of the human OPG polypeptide was placed under control of the luxPR promoter in the plasmid expression vector pAMG21 as follows. To accomplish this, PCR using oligonucleotides #1257-20 and #1257-19 as primers was performed using as a template plasmid pRcCMV-Hu OPG DNA containing the human OPG cDNA and thermocycling for 30 cycles with each cycle being: 94°C for 20 seconds, followed by 37°C for 30 seconds, followed by 72°C for 30 seconds. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, and restricted with KpnI and BamHI restriction endonucleases and purified. Synthetic oligonucleotides #1257-21 and #1257-22 were phosphorylated individually using T4 polynucleotide kinase and ATP, and were then mixed together, heated at 94°C and allowed to slow cool to room temperature to form an oligonucleotide linker duplex containing NdeI and KpnI sticky ends. The phosphorylated linker duplex formed between oligonucleotides #1257-21 and #1257-22 containing NdeI and KpnI cohesive ends (see Figure 14A) and the KpnI and BamHI digested and purified PCR product generated



using oligo primers #1257-20 and #1257-19 (see above)  
was directionally inserted between two sites of the  
plasmid vector pAMG21, namely the NdeI site and BamHI  
site, using standard recombinant DNA methodology (see  
5 Figure 14A and sequences below). The synthetic linker  
utilized E. coli codons and provided for a N-terminal  
methionine.

Two clones were selected and plasmid DNA  
isolated, and the human OPG insert was subsequently DNA  
10 sequence confirmed. The resulting pAMG21 plasmid  
containing amino acids 32-401 of the human OPG  
polypeptide immediately preceded in frame by a  
methionine is referred to as pAMG21-huOPG met[32-401]  
or pAMG21-huOPG met[32-401].

15

Oligo#1257-19

5'-TACGCACTGGATCCTTATAAGCAGCTTATTTTACTGATTGGAC-3'

(SEQ ID NO:59)

20

Oligo#1257-20

5'-GTCCTCCTGGTACCTACCTAAAACAAC-3' (SEQ ID NO:60)

Oligo#1257-21

5'-TATGGATGAAGAACTTCTCATCAGCTGCTGTGTGATAAATGTCC

25

GCCGGGTAC -3' (SEQ ID NO:61)

Oligo#1257-22

5'-CCGGCGGACATTTATCACACAGCAGCTGATGAGAAGTTTCTTCATCCA-3'

(SEQ ID NO:47)

30

Cultures of pAMG21-huOPG met[32-401] in E.  
coli GM120 in 2XYT media containing 20 µg/ml kanamycin  
were incubated at 30°C prior to induction. Induction  
of huOPG met[32-401] gene product expression from the  
35 luxPR promoter was achieved following the addition of  
the synthetic autoinducer N-(3-oxohexanoyl)-DL-

homoserine lactone to the culture media to a final concentration of 30 ng/ml and cultures were incubated at either 30°C or 37°C for a further 6 hours. After 6 hours, the bacterial cultures were examined by

5 microscopy for the presence of inclusion bodies and were then pelleted by centrifugation. Refractile inclusion bodies were observed in induced cultures indicating that some of the recombinant huOPG met[32-401] gene product was produced insolubly in E. coli.

10 Some bacterial pellets were resuspended in 10mM Tris-HCl/pH8, 1mM EDTA and lysed directly by addition of 2X Laemlli sample buffer to 1X final, and  $\beta$ -mercaptoethanol to 5% final concentration, and analyzed by SDS-PAGE. A substantially more intense coomassie

15 stained band of approximately 42kDa was observed on a SDS-PAGE gel containing total cell lysates of 30°C and 37°C induced cultures versus lane 2 which is a total cell lysate of a 30°C uninduced culture (Figure 14B). The expected gene product would be 370 amino acids in

20 length and have an expected molecular weight of about 42.2 kDa. Following induction at 37°C for 6 hours, an additional culture was pelleted and either processed for isolation of inclusion bodies (see below) or processed by microfluidizing. The pellet processed for

25 microfluidizing was resuspended in 25mM Tris-HCl/pH8, 0.5M NaCl buffer and passed 20 times through a Microfluidizer Model 1108 (Microfluidics Corp.) and collected. An aliquot was removed of the collected sample (microfluidized total lysate), and the remainder

30 was pelleted at 20,000 x g for 20 minutes. The supernatant following centrifugation was removed (microfluidized soluble fraction) and the pellet resuspended in a 25mM Tris-HCl/pH8, 0.5M NaCl, 6M urea solution (microfluidized insoluble fraction). To an

35 aliquot of either the total soluble, or insoluble fraction was added to an equal volume of 2X Laemalli

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sample buffer and  $\beta$ -mercaptoethanol to 5% final concentration. The samples were then analyzed by SDS-PAGE. A significant amount of recombinant huOPG met[32-401] gene product appeared to be found in the insoluble fraction. To purify the recombinant protein inclusion bodies were purified as follows: Bacterial cells were separated from media by density gradient centrifugation in a Beckman J-6B centrifuge equipped with a JS-4.2 rotor at 4,900 x g for 15 minutes at 4°C. The bacterial pellet was resuspended in 5 ml of water and then diluted to a final volume of 10 ml with water. This suspension was transferred to a stainless steel cup cooled in ice and subjected to sonic disruption using a Branson Sonifier equipped with a standard tip (power setting=5, duty cycle=95%, 80 bursts). The sonicated cell suspension was centrifuged in a Beckman Optima TLX ultracentrifuge equipped with a TLA 100.3 rotor at 195,000 x g for 5 to 10 minutes at 23°C. The supernatant was discarded and the pellet rinsed with a stream of water from a squirt bottle. The pellets were collected by scraping with a micro spatula and transferred to a glass homogenizer (15 ml capacity). Five ml of Percoll solution (75% liquid Percoll, 0.15 M sodium chloride) was added to the homogenizer and the contents are homogenized until uniformly suspended. The volume was increased to 19.5 ml by the addition of Percoll solution, mixed, and distributed into 3 Beckman Quick-Seal tubes (13 x 32 mm). Tubes were sealed according to manufacturers instructions. The tubes were spun in a Beckman TLA 100.3 rotor at 23°C, 20,000 rpm (21,600 x g), 30 minutes. The tubes were examined for the appropriate banding pattern. To recover the refractile bodies, gradient fractions were recovered and pooled, then diluted with water. The inclusion

bodies were pelleted by centrifugation, and the protein concentration estimated following SDS-PAGE.

An aliquot of inclusion bodies isolated as described below was dissolved into 1X Laemmli sample  
5 buffer with 5%  $\beta$ -mercaptoethanol and resolved on a SDS-PAGE gel and the isolated inclusion bodies provide a highly purified recombinant huOPG[32-401] gene product. The major ~42 kDa band observed after resolving  
10 inclusion bodies on a SDS-polyacrylamide gel was excised from a separate gel and the N-terminal amino acid sequence determined essentially as described (Matsudaira et al. J. Biol. Chem. 262, 10-35 (1987)). The following sequence was determined after 19 cycles:

15 NH<sub>2</sub> -MDEETSHQLLCDKCPPGTY-COOH (SEQ ID NO:62)

This sequence was found to be identical to the first 19 amino acids encoded by the pAMG21 Hu-OPG met[32-401] expression vector, produced by a methionine residue  
20 provided by the bacterial expression vector.

#### C. Human OPG met[22-401]

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG  
25 was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. Isolated plasmid DNA of pAMG21-huOPG met[32-401] (see Section B) was cleaved with KpnI and BamHI restriction endonucleases and the resulting fragments  
30 were resolved on an agarose gel. The B fragment (~1064 bp fragment) was isolated from the gel using standard methodology. Synthetic oligonucleotides (oligos) #1267-06 and #1267-07 were phosphorylated individually and allowed to form an oligo linker duplex, which  
35 contained NdeI and KpnI cohesive ends, using methods described in Section B. The synthetic linker duplex

utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated oligo linker containing NdeI and KpnI cohesive ends and the isolated ~1064 bp fragment of pAMG21-huOP met[32-401] digested with KpnI and BamHI restriction endonucleases were directionally inserted between the NdeI and BamHI sites of pAMG21 using standard recombinant DNA methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[22-401] gene.

Oligo #1267-06

5'-TAT GGA AAC TTT TCC TCC AAA ATA TCT TCA TTA TGA TGA  
AGA AAC TTC TCA TCA GCT GCT GTG TGA TAA ATG TCC GCC GGG  
TAC-3' (SEQ ID NO:63)

Oligo #1267-07

5'-CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAG AAG TTT  
CTT CAT CAT AAT GAA GAT ATT TTG GAG GAA AAG TTT CCA-3'  
(SEQ ID NO:64)

Cultures of pAMG21-huOPG-met[22-401] in E. coli host 393 were placed in 2XYT media containing 20 µg/ml kanamycin and were incubated at 30°C prior to induction. Induction of recombinant gene product expression from the luxPR promoter of vector pAMG21 was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 30 ng/ml and incubation at either 30°C or 37°C for a further 6 hours. After 6 hours, bacterial cultures were pelleted by centrifugation (=30°C I+6 or 37°C I+6). Bacterial cultures were also either pelleted just prior to induction (=30°C PreI) or alternatively no autoinducer

was added to a separate culture which was allowed to incubate at 30°C for a further 6 hours to give an uninduced (UI) culture (=30°C UI). Bacterial pellets of either 30°C PreI, 30°C UI, 30°C I+6, or 37°C I+6 cultures were resuspended, lysed, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described in Section B. Polyacrylamide gels were either stained with coomassie blue and/or Western transferred to nitrocellulose and immunoprobed with rabbit anti-mu OPG-Fc polyclonal antibody as described in Example 10. The level of gene product following induction compared to either an uninduced (30°C UI) or pre-induction (30°C PreI) sample.

D. Murine OPG met[22-401]

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of the murine (mu) OPG (OPG) polypeptide was placed under control of the luxPR promoter in a prokaryotic plasmid expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1257-16 and #1257-15 as primers, plasmid pRcCMV-Mu OPG DNA as a template and thermocycling conditions as described in Section B. The PCR product was purified and cleaved with KpnI and BamHI restriction endonucleases as described in Section B. Synthetic oligos #1260-61 and #1260-82 were phosphorylated individually and allowed to form an oligo linker duplex with NdeI and KpnI cohesive ends using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed between oligos #1260-61 and #1260-82 containing NdeI and KpnI cohesive ends and the KpnI and BamHI digested and purified PCR product generated using oligo primers #1257-16 and #1257-15 were directionally inserted between the NdeI and BamHI sites of pAMG21

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GAT TGA AC-3' (SEQ ID NO:65)

TG-3' (SEQ ID NO:66)

20

5' -TAT GGA AAC TCT GCC TCC AAA ATA CCT GCA TTA CGA  
TCC GGA AAC TGG TCA TCA GCT GCT GTG TGA TAA ATG TGC TCC  
GGG TAC-3' (SEQ ID NO:67)

25

TTT CCG GAT CGT AAT GCA GGT ATT TTG GAG GCA GAG TTT  
CCA-3' (SEQ ID NO:68)

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allowed to form an oligo linker duplex using methods described in Section B. The synthetic linker duplex utilized E. coli codons and provided for an N-terminal methionine. The phosphorylated linker duplex formed  
5 between oligos #1267-08 and #1267-09 containing NdeI and KpnI cohesive ends, and the KpnI and BamHI digested and purified PCR product described earlier (see Section D), was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The  
10 ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[32-401] gene.

15 Expression of recombinant muOPG-met [32-401] polypeptide from cultures of 393 cells harboring the pAMG21 recombinant plasmid following induction was determined using methods described in Section C.

20 Oligo #1267-08

5'-TAT GGA CCC AGA AAC TGG TCA TCA GCT GCT GTG TGA  
TAA ATG TGC TCC GGG TAC-3' (SEQ ID NO:69)

Oligo #1267-09

25 5'-CCG GAG CAC ATT TAT CAC ACA GCA GCT GAT GAC CAG  
TTT CTG GGT CCA-3' (SEQ ID NO:70)

F. Murine OPG met-lys[22-401]

A DNA sequence coding for an N-terminal  
30 methionine followed by a lysine residue and amino acids 22 through 401 of murine OPG was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as follows. Synthetic oligos #1282-95 and #1282-96 were phosphorylated individually and allowed  
35 to form an oligo linker duplex using methods described in Section B. The synthetic linker duplex utilized E.



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Oligo #1282-95

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Oligo #1282-96

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G. Murine OPG met-lys-(his)<sub>7</sub>[22-401]

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B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, cleaved with NdeI and BamHI restriction endonucleases and purified. The NdeI and BamHI digested and purified PCR product generated using oligo primers #1300-50 and #1257-15 was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard DNA methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing performed to verify the DNA sequence of the muOPG-MKH[22-401] gene.

Expression of recombinant MuOPG-MKH[22-401] polypeptide from transformed 393 cultures harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1300-50

5'-GTT CTC CTC ATA TGA AAC ATC ATC ACC ATC ACC ATC  
ATG AAA CTC TGC CTC CAA AAT ACC TGC ATT ACG AT-3' (SEQ  
ID NO:73)

Oligo #1257-15

(see Section D)

#### H. Murine OPG met-lys[22-401](his)<sub>7</sub>

A DNA sequence coding for a N-terminal met-lys, amino acids 22 through 401 murine OPG, and seven histidine residues following amino acid 401 (=muOPG MK[22-401]-H<sub>7</sub>), was placed under control of the lux PR promoter in prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1300-49 and #1300-51 as primers and pAMG21-muOPG met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product

was excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product was directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG MK[22-401]-H7 gene.

Expression of the recombinant muOPG MK-[22-401]-H7 polypeptide from a transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1300-49

5'-GTT CTC CTC ATA TGA AAG AAA CTC TGC CTC CAA AAT  
ACC TGC A-3' (SEQ ID NO:74)

Oligo #1300-51

5'-TAC GCA CTG GAT CCT TAA TGA TGG TGA TGG TGA TGA  
TGT AAG CAG CTT ATT TTC ACG GAT TGA ACC TGA TTC CCT A-  
3' (SEQ ID NO:75)

#### I. Murine OPG met[27-401]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed with oligonucleotides #1309-74 and #1257-15 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, cleaved with NdeI and BamHI

restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[27-401] gene.

Expression of recombinant muOPG-met[27-401] polypeptide from a transfected 393 culture harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo#1309-74

5'-GTT CTC CTC ATA TGA AAT ACC TGC ATT ACG ATC CGG  
AAA CTG GTC AT-3' (SEQ ID NO:76)

Oligo#1257-15

(See Section D)

#### J. Human OPG met[27-401]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 401 of human OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1309-75 and #1309-76 as primers and plasmid pAMG21-huOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, restricted with AseI and BamHI restriction endonucleases, and purified. The AseI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation

mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[27-401] gene.

Expression of the recombinant huOPG-met[27-401] polypeptide following induction of from transfected 393 cells harboring the recombinant pAMG21 plasmid was determined using methods described in Section C.

Oligo #1309-75

5'-GTT CTC CTA TTA ATG AAA TAT CTT CAT TAT GAT GAA  
GAA ACT T-3' (SEQ ID NO:77)

Oligo #1309-76

5'-TAC GCA CTG GAT CCT TAT AAG CAG CTT ATT TTT ACT  
GAT T-3' (SEQ ID NO:78)

## K. Murine OPG met[22-180]

A DNA sequence coding for a N-terminal methionine and amino acids 22 through 180 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed with oligonucleotides #1309-72 and #1309-73 as primers and plasmid pAMG21-muOPG-met[22-401] DNA as template. Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product was excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product above was directionally inserted between the NdeI and BamHI sites of pAMG21 using standard methodology. The ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were

selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG-met[22-180] gene.

Expression of recombinant muOPG-met[22-180] polypeptide from transformed 393 cultures harboring the recombinant pAMG21 plasmid following induction was determined using methods described in Section C.

Oligo #1309-72

10 5'-GTT CTC CTC ATA TGG AAA CTC TGC CTC CAA AAT ACC  
TGC A-3' (SEQ ID NO:79)

Oligo #1309-73

15 5'-TAC GCA CTG GAT CCT TAT GTT GCA TTT CCT TTC TGA  
ATT AGC A-3' (SEQ ID NO:80)

L. Murine OPG met[27-180]

A DNA sequence coding for a N-terminal methionine and amino acids 27 through 180 of murine OPG was placed under the control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. PCR was performed using oligonucleotides #1309-74 (see Section I) and #1309-73 (see Section K) as primers and plasmid pAMG21-muOPG met[22-401] DNA as template.

25 Thermocycling conditions were as described in Section B. The resulting PCR sample was resolved on an agarose gel, the PCR product excised, purified, restricted with NdeI and BamHI restriction endonucleases, and purified. The NdeI and BamHI digested and purified PCR product

30 above was directionally inserted between the NdeI and BamHI sites in pAMG21 using standard methodology. The ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was

35 isolated, and DNA sequencing was performed to verify the DNA sequence of the muOPG met[27-180] gene.

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Expression of recombinant muOPG met[27-180] polypeptide from cultures of transformed 393 cells harboring the recombinant pAMG21 plasmid following induction was determined using methods described in  
5 Section C.

M. Murine OPG met[22-189] and met[22-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 22 through 189, or 22  
10 through 194 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. The pair of synthetic oligonucleotides #1337-92 and #1337-93 (=muOPG-189 linker) or #1333-57 and #1333-58 (=muOPG-194 linker)  
15 were phosphorylated individually and allowed to form an oligo linker duplex pair using methods described in Section B. Purified plasmid DNA of pAMG21-muOPG-met[22-401] was cleaved with KpnI and BspEI restriction endonucleases and the resulting DNA fragments were  
20 resolved on an agarose gel. The ~413 bp B fragment was isolated using standard recombinant DNA methodology. The phosphorylated oligo linker duplexes formed between either oligos #1337-92 and #1337-93 (muOPG-189 linker) or oligos #1333-57 and #1333-58 (muOPG-194 linker)  
25 containing BspEI and BamHI cohesive ends, and the isolated ~413 bp B fragment of plasmid pAMG21-muOPG-met[22-401] digested with KpnI and BspEI restriction endonucleases above, was directionally inserted between the KpnI and BamHI sites of pAMG21-muOPG met[22-401]  
30 using standard methodology. Each ligation mixture was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of either the  
35 muOPG-met[22-189] or muOPG-met[22-194] genes.

Expression of recombinant muOPG-met[22-189] and muOPG-met[22-194] polypeptides from recombinant pAMG21 plasmids transformed into 393 cells was determined using methods described in Section C.

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Oligo #1337-92

5'-CCG GAA ACA GAT AAT GAG-3' (SEQ ID NO:81)

Oligo #1337-93

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5'-GAT CCT CAT TAT CTG TTT-3' (SEQ ID NO:82)

Oligo #1333-57

5'-CCG GAA ACA GAG AAG CCA CGC AAA AGT AAG-3'  
(SEQ ID NO:83)

15

Oligo #1333-58

5'-GAT CCT TAC TTT TGC GTG GCT TCT CTG TTT-3'  
(SEQ ID NO:84)

20

N. Murine OPG met[27-189] and met[27-194]

A DNA sequence coding for a N-terminal methionine and either amino acids 27 through 189, or 27 through 194 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. Phosphorylated oligo linkers either "muOPG-189 linker" or "muOPG-194 linker" (see Section M) containing BspEI and BamHI cohesive ends, and the isolated ~413 bp B fragment of plasmid pAMG21-muOPG-met[22-401] digested with KpnI and BspEI restriction endonucleases were directionally inserted between the KpnI and BamHI sites of plasmid pAMG21-muOPG-met[27-401] using standard methodology. Each ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing

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was performed to verify the DNA sequence of either the muOPG met[27-189] or muOPG met[27-194] genes.

Expression of recombinant muOPG met[27-189] and muOPG met[27-194] following induction of 393 cells  
5 harboring recombinant pAMG21 plasmids was determined using methods described in Section C.

O. Human OPG met[22-185], met[22-189], met[22-194]

A DNA sequence coding for a N-terminal  
10 methionine and either amino acids 22 through 185, 22 through 189, or 22 through 194 of the human OPG polypeptide was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. The pair of synthetic oligonucleotides #1331-  
15 87 and #1331-88 (=huOPG-185 linker), #1331-89 and #1331-90 (=huOPG-189 linker), or #1331-91 & #1331-92 (=huOPG-194 linker) were phosphorylated individually and each allowed to form an oligo linker duplex pair using methods described in Section B. Purified plasmid  
20 DNA of pAMG21-huOPG-met[27-401] was restricted with KpnI and NdeI restriction endonucleases and the resulting DNA fragments were resolved on an agarose gel. The ~407 bp B fragment was isolated using standard recombinant DNA methodology. The  
25 phosphorylated oligo linker duplexes formed between either oligos #1331-87 and #1331-88 (huOPG-185 linker), oligos #1331-89 and #1331-90 (huOPG-189 linker), or oligos #1331-91 and #1331-92 (huOPG-194 linker)[each linker contains NdeI and BamHI cohesive ends], and the  
30 isolated ~407 bp B fragment of plasmid pAMG21-huOPG-met[27-401] digested with KpnI and NdeI restriction endonucleases above, was directionally inserted between the KpnI and BamHI sites of plasmid pAMG21-huOPG-met[22-401] using standard methodology. Each ligation  
35 was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol.

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Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of either the huOPG-met[22-185], huOPG-met[22-189], or huOPG-met[22-194] genes.

5            Expression of recombinant huOPG-met[22-185], huOPG-met[22-189] or huOPG-met[22-194] in transformed 393 cells harboring recombinant pAMG21 plasmids following induction was determined using methods described in Section C.

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    Oligo #1331-87

    5'-TAT GTT AAT GAG-3' (SEQ ID NO:85)

    Oligo #1331-88

15

    5'-GAT CCT CAT TAA CA-3' (SEQ ID NO:86)

    Oligo #1331-89

    5'-TAT GTT CCG GAA ACA GTT AAG-3' (SEQ ID NO:87)

20

    Oligo #1331-90

    5'-GAT CCT TAA CTG TTT CCG GAA CA-3' (SEQ ID NO:88)

    Oligo #1331-91

25

    5'-TAT GTT CCG GAA ACA GTG AAT CAA CTC AAA AAT AAG-3' (SEQ ID NO:89)

    Oligo #1331-92

30      5'-GAT CCT TAT TTT TGA GTT GAT TCA CTG TTT CCG GAA CA-3' (SEQ ID NO:90)

P. Human OPG met[27-185], met[27-189], met [27-194]

35            A DNA sequence coding for a N-terminal methionine and either amino acids 27 through 185, 27 through 189, or 27 through 194 of the human OPG

polypeptide was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as follows. Phosphorylated oligo linkers "huOPG-185 linker", "huOPG-189 linker", or "huOPG-194 linker" (See  
5 Section O) each containing NdeI and BamHI cohesive ends, and the isolated ~407 bp B fragment of plasmid pAMG21-huOPG-met[27-401] digested with KpnI and NdeI restriction endonucleases (See Section O) were directionally inserted between the KpnI and BamHI sites  
10 of plasmid pAMG21-huOPG-met[27-401] (See Section J) using standard methodology. Each ligation was transformed into E. coli host 393 by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA isolated, and DNA sequencing  
15 performed to verify the DNA sequence of either the huOPG-met[27-185], huOPG-met[27-189], or huOPG-met[27-194] genes.

Expression of recombinant huOPG-met[27-185], huOPG-met[27-189], and huOPG-met[27-194] from  
20 recombinant pAMG21 plasmids transformed into 393 cells was determined using methods described in Section C.

Q. Murine OPG met[27-401] (P33E, G36S, A45P)

25 A DNA sequence coding for an N-terminal methionine and amino acids 27 through 48 of human OPG followed by amino acid residues 49 through 401 of murine OPG was placed under control of the lux PR promoter of prokaryotic expression vector pAMG21 as  
30 follows. Purified plasmid DNA of pAMG21-huOPG-met[27-401] (See Section J) was cleaved with AatII and KpnI restriction endonucleases and a ~1075 bp B fragment isolated from an agarose gel using standard recombinant DNA methodology. Additionally, plasmid pAMG21-muOPG-  
35 met[22-401] DNA (See Section D) was digested with KpnI and BamHI restriction endonucleases and the ~1064 bp B

fragment isolated as described above. The isolated  
~1075 bp pAMG21-huOPG-met[27-401] restriction fragment  
containing AatII & KpnI cohesive ends (see above), the  
~1064 bp pAMG21-muOPG-met[22-401] restriction fragment  
5 containing KpnI and BamHI sticky ends and a ~5043 bp  
restriction fragment containing AatII and BamHI  
cohesive ends and corresponding to the nucleic acid  
sequence of pAMG21 between AatII & BamHI were ligated  
using standard recombinant DNA methodology. The  
10 ligation was transformed into *E. coli* host 393 by  
electroporation utilizing the manufacturer's protocol.  
Clones were selected, and the presence of the  
recombinant insert in the plasmid verified using  
standard DNA methodology. muOPG-27-401 (P33E, G36S,  
15 A45P) gene. Amino acid changes in muOPG from proline-  
33 to glutamic acid-33, glycine-36 to serine-36, and  
alanine-45 to proline-45, result from replacement of  
muOPG residues 27 through 48 with huOPG residues 27  
through 48.

20 Expression of recombinant muOPG-met[27-401]  
(P33E, G36S, A45P) from transformed 393 cells harboring  
the recombinant pAMG21 plasmid was determined using  
methods described in Section C.

25 R. Murine OPG met-lys-(his)<sub>7</sub>-ala-ser-(asp)<sub>4</sub>-lys[22-401]  
(A45T)

A DNA sequence coding for an N-terminal His  
tag and enterokinase recognition sequence which is (NH<sub>2</sub>  
30 to COOH terminus): Met-Lys-His-His-His-His-His-  
His-Ala-Ser-Asp-Asp-Asp-Asp-Lys (=HEK), followed by  
amino acids 22 through 401 of the murine OPG  
polypeptide was placed under control of the lac  
repressor regulated Ps4 promoter as follows. pAMG22-  
35 His (See Section A) was digested with NheI and BamHI  
restriction endonucleases, and the large fragment (the

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S. Human OPG met-arg-gly-ser-(his)<sub>6</sub>[22-401]

Eight oligonucleotides (1338-09 to 1338-16 shown below) were designed to produce a 175 base fragment as overlapping, double stranded DNA. The oligos were annealed, ligated, and the 5' and 3' oligos were used as PCR primers to produce large quantities of the 175 base fragment. The final PCR gene products were digested with restriction endonucleases ClaI and KpnI to yield a fragment which replaces the N-terminal 28 codons of human OPG. The ClaI and KpnI digested PCR product was inserted into pAMG21-huOPG [27-401] which had also been cleaved with ClaI and KpnI. Ligated DNA was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. Expression of huOPG Met-Arg-Gly-Ser-(His)<sub>6</sub> [22-401] resulting in the formation of large inclusion bodies and the protein was localized to the insoluble (pellet) fraction.

1338-09

ACA AAC ACA ATC GAT TTG ATA CTA GA (SEQ ID NO:93)

1338-10

TTT GTT TTA ACT AAT TAA AGG AGG AAT AAA ATA TGA GAG GAT CGC ATC AC  
(SEQ ID NO:94)

1338-11

CAT CAC CAT CAC GAA ACC TTC CCG CCG AAA TAC CTG CAC TAC GAC GAA GA  
(SEQ ID NO:95)

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AAC CTC CCA CCA GCT GCT GTG CGA CAA ATG CCC GCC GGG TAC CCA AAC A  
(SEQ ID NO:96)

1338-13

TGT TTG GGT ACC CGG CGG GCA TTT GT (SEO ID NO:97)

10 CGC ACA GCA GCT GGT GGG AGG TTT CTT CGT CGT AGT GCA GGT ATT TCG GC  
(SEQ ID NO:98)

GGG AAG GTT TCG TGA TGG TGA TGG TGA TGC GAT CCT CTC ATA TTT TAT T  
15 (SEQ ID NO:99)

CCT CCT TTA ATT AGT TAA AAC AAA TCT AGT ATC AAA TCG ATT GTG TTT GT  
(SEQ ID NO:100)

T. Human OPG met-lys[22-401] and met(lys)<sub>3</sub>[22-401]

To construct the met-lys and met-(lys)<sub>3</sub> versions of human OPG[22-401], overlapping oligonucleotides were designed to add the appropriate number of lysine residues. The two oligos for each construct were designed to overlap, allowing two rounds of PCR to produce the final product. The template for the first PCR reaction was a plasmid DNA preparation containing the human OPG 22-401 gene. The first PCR added the lysine residue(s). The second PCR used the product of the first round and added sequence back to the first restriction site, ClaI.

The final PCR gene products were digested with restriction endonucleases ClaI and KpnI, which replace the N-terminal 28 codons of hu OPG, and then ligated into plasmid pAMG21-hu OPG [27-401] which had

been also digested with the two restriction  
endonucleases. Ligated DNA was transformed into  
competent host cells of E. coli strain 393. Clones  
were screened for the ability to produce the  
5 recombinant protein product and to possess the gene  
fusion having the correct nucleotide sequence. Protein  
expression levels were determined from 50 ml shaker  
flask studies. Whole cell lysate and sonic pellet were  
analyzed for expression of the construct by Coomassie  
10 stained PAGE gels and Western analysis with murine  
anti-OPG antibody. Neither construct had a detectable  
level of protein expression and inclusion bodies were  
not visible. The DNA sequences were confirmed by DNA  
sequencing.

15 Oligonucleotide primers to prepare Met-Lys huOPG[22-  
401]:  
1338-17  
ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT  
20 AAA GGA GGA ATA AAA TG (SEQ ID NO:101)

1338-18  
CTA ATT AAA GGA GGA ATA AAA TGA AAG AAA CTT TTC CTC CAA  
AAT ATC (SEQ ID NO:102)

25 1338-20  
TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID  
NO:103)

30 Oligonucleotide primers to prepare Met-(Lys)<sub>3</sub>-huOPG[22-  
401]:  
1338-17  
ACA AAC ACA ATC GAT TTG ATA CTA GAT TTG TTT TAA CTA ATT  
AAA GGA GGA ATA AAA TG (SEQ ID NO:104)

35



1338-19

CTA ATT AAA GGA GGA ATA AAA TGA AAA AAA AAG AAA CTT TTC  
CTC CAA AAT ATC (SEQ ID NO:105)

5 1338-20

TGT TTG GGT ACC CGG CGG ACA TTT ATC ACA C (SEQ ID  
NO:106)

U. Human and Murine OPG [22-401]/Fc Fusions

10 Four OPG-Fc fusions were constructed where  
the Fc region of human IgG1 was fused at the N-terminus  
of either human or murine Osteoprotegerin amino acids  
22 to 401 (referred to as Fc/OPG [22-401]) or at the C-  
terminus (referred to as OPG[22-401]/Fc). Fc fusions  
15 were constructed using the fusion vector pFc-A3  
described in Example 7.

All fusion genes were constructed using  
standard PCR technology. Template for PCR reactions  
were plasmid preparations containing the target genes.  
20 Overlapping oligos were designed to combine the  
C-terminal portion of one gene with the N terminal  
portion of the other gene. This process allows fusing  
the two genes together in the correct reading frame  
after the appropriate PCR reactions have been  
25 performed. Initially one "fusion" oligo for each gene  
was put into a PCR reaction with a universal primer for  
the vector carrying the target gene. The complimentary  
"fusion" oligo was used with a universal primer to PCR  
the other gene. At the end of this first PCR reaction,  
30 two separate products were obtained, with each  
individual gene having the fusion site present,  
creating enough overlap to drive the second round of  
PCR and create the desired fusion. In the second round  
of PCR, the first two PCR products were combined along  
35 with universal primers and via the overlapping regions,  
the full length fusion DNA sequence was produced.

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The final PCR gene products were digested with restriction endonucleases XbaI and BamHI, and then ligated into the vector pAMG21 having been also digested with the two restriction endonucleases.

- 5 Ligated DNA was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were  
10 determined from 50 ml shaker flask studies. Whole cell lysate, sonic pellet, and supernatant were analyzed for expression of the fusion by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody.

15 Fc/huOPG [22-401]

- Expression of the Fc/hu OPG [22-401] fusion peptide was detected on a Coomassie stained PAGE gel and on a Western blot. The cells have very large inclusion bodies, and the majority of the product is in  
20 the insoluble (pellet) fraction. The following primers were used to construct this OPG-Fc fusion:

1318-48

- 25 CAG CCC GGG TAA AAT GGA AAC GTT TCC TCC AAA ATA TCT TCA  
TT (SEQ ID NO:107)

1318-49

- CGT TTC CAT TTT ACC CGG GCT GAG CGA GAG GCT CTT CTG CGT  
30 GT (SEQ ID NO:108)

Fc/muOPG [22-401]

- Expression of the fusion peptide was detected on a Coomassie stained gel and on a Western blot. The  
35 cells have very large inclusion bodies, and the majority of the product is in the insoluble (pellet)

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fraction. The following primers were used to construct this OPG-Fc fusion:

1318-50

5 CGC TCA GCC CGG GTA AAA TGG AAA CGT TGC CTC CAA AAT ACC  
TGC (SEQ ID NO:109)

1318-51

CCA TTT TAC CCG GGC TGA GCG AGA GGC TCT TCT GCG TGT  
10 (SEQ ID NO:110)

muOPG [22-401]/Fc

Expression of the fusion peptide was detected on a Coomassie stained gel and on a Western blot. The  
15 amount of recombinant product was less than the OPG  
fusion proteins having the Fc region in the N terminal  
position. Obvious inclusion bodies were not detected.  
Most of the product appeared to be in the insoluble  
(pellet) fraction. The following primers were used to  
20 construct this OPG-Fc fusion:

1318-54

GAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC  
(SEQ ID NO:111)

25

1318-55

CAG CTG CAG CTA AGC AGC TTA TTT TCA CGG ATT G  
(SEQ ID NO:112)

30

huOPG [22-401]/Fc

Expression of the fusion peptide was not detected on a Coomassie stained gel, although a faint  
Western positive signal was present. Obvious inclusion  
35 bodies were not detected. The following primers were  
used to prepare this OPG-Fc fusion:

AAA AAT AAG CTG CTT AGC TGC AGC TGA ACC AAA ATC  
(SEQ ID NO:113)

1318-53

CAG CTG CAG CTA AGC AGC TTA TTT TTA CTG ATT GG  
(SEQ ID NO:114)

10 V. Human OPG met[22-401]-Fc fusion (P25A)

This construct combines a proline to alanine amino acid change at position 25 (P25A) with the huOPG met[22-401]-Fc fusion. The plasmid was digested with restriction endonucleases ClaI and KpnI, which removes the N-terminal 28 codons of the gene, and the resulting small (less than 200 base pair) fragment was gel purified. This fragment containing the proline to alanine change was then ligated into plasmid pAMG21-huOPG [22-401]-Fc fusion which had been digested with the two restriction endonucleases. The ligated DNA was transformed into competent host cells of E. coli strain 393. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having the correct nucleotide sequence. Protein expression levels were determined from 50 ml shaker flask studies. Whole cell lysate and sonic pellet were analyzed for expression of the construct by Coomassie stained PAGE gels and Western analysis with murine anti-OPG antibody. The expression level of the fusion peptide was detected on a Coomassie stained PAGE gel and on a Western blot. The protein was in the insoluble (pellet) fraction. The cells had large inclusion bodies.

W. Human OPG met[22-401] (P25A)

5 A DNA sequence coding for an N-terminal  
methionine and amino acids 22 through 401 of human OPG  
with the proline at position 25 being substituted by  
alanine under control of the lux Pr promoter in  
prokaryotic expression vector pAMG21 was constructed as  
follows: Synthetic oligos # 1289-84 and 1289-85 were  
annealed to form an oligo linker duplex with XbaI and  
KpnI cohesive ends. The synthetic linker duplex  
10 utilized optimal E. coli codons and encoded an N-  
terminal methionine. The linker also included an SpeI  
restriction site which was not present in the original  
sequence. The linker duplex was directionally inserted  
between the XbaI and KpnI sites in pAMG21-huOPG-22-401  
15 using standard methods. The ligation mixture was  
introduced into E. coli host GM221 by transformation.  
Clones were initially screened for production of the  
recombinant protein. Plasmid DNA was isolated from  
positive clones and DNA sequencing was performed to  
20 verify the DNA sequence of the HuOPG-Met[22-401] (P25A)  
gene. The following oligonucleotides were used to  
generate the XbaI - KpnI linker:

Oligo #1289-84

25 5'-CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TGC TCC  
AAA ATA TCT TCA TTA TGA TGA AGA AAC TAG TCA TCA GCT GCT  
GTG TGA TAA ATG TCC GCC GGG TAC -3' (SEQ ID NO:115)

30 Oligo #1289-85

5'- CCG GCG GAC ATT TAT CAC ACA GCA GCT GAT GAC  
TAG TTT CTT CAT CAT AAT GAA GAT ATT TTG GAG CAA AAG TTT  
CCA TAT GTT ATT CCT CCT T-3' (SEQ ID NO:116)

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X. Human OPG met[22-401] (P26A) and (P26D)

A DNA sequence coding for an N-terminal methionine and amino acids 22 through 401 of human OPG with the proline at position 26 being substituted by alanine under control of the lux P<sub>R</sub> promoter in prokaryotic expression vector pAMG21 was constructed as follows: Synthetic oligos # 1289-86 and 1289-87 were annealed to form an oligo linker duplex with XbaI and SpeI cohesive ends. The synthetic linker duplex utilized optimal E. coli codons and encoded an N-terminal methionine. The linker duplex was directionally inserted between the XbaI and SpeI sites in pAMG21-huOPG[22-401](P25A) using standard methods. The ligation mixture was introduced into E. coli host GM221 by transformation. Clones were initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing was performed to verify the DNA sequence of the huOPG-met[22-401](P26A) gene. One of the clones sequenced was found to have the proline at position 26 substituted by aspartic acid rather than alanine, and this clone was designated huOPG-met[22-401](P26D). The following oligonucleotides were used to generate the XbaI - SpeI linker:

Oligo #1289-86

5' - CTA GAA GGA GGA ATA ACA TAT GGA AAC TTT TCC  
TGC TAA ATA TCT TCA TTA TGA TGA AGA AA - 3'  
(SEQ ID NO:117)

Oligo #1289-87

5' - CTA GTT TCT TCA TCA TAA TGA AGA TAT TTA GCA  
GGA AAA GTT TCC ATA TGT TAT TCC TCC TT - 3'  
(SEQ ID NO:118)

Y. Human OPG met[22-194] (P25A)

5 A DNA sequence coding for an N-terminal methionine and amino acids 22 through 194 of human OPG with the proline at position 25 being substituted by alanine under control of the lux Pr promoter in prokaryotic expression vector pAMG21 was constructed as follows: The plasmids pAMG21-huOPG[27-194] and pAMG21-huOPG[22-401] (P25A) were each digested with KpnI and  
10 BamHI endonucleases. The 450 bp fragment was isolated from pAMG21-huOPG[27-194] and the 6.1 kbp fragment was isolated from pAMG21-huOPG[22-401] (P25A). These fragments were ligated together and introduced into E. coli host GM221 by transformation. Clones were  
15 initially screened for production of the recombinant protein. Plasmid DNA was isolated from positive clones and DNA sequencing was performed to verify the DNA sequence of the huOPG-Met[22-194] (P25A) gene.

20 EXAMPLE 9

Association of OPG Monomers

CHO cells engineered to overexpress muOPG [22-401] were used to generate conditioned media for  
25 the analysis of secreted recombinant OPG using rabbit polyclonal anti-OPG antibodies. An aliquot of conditioned media was concentrated 20-fold, then analysed by reducing and non-reducing SDS-PAGE (Figure 15). Under reducing conditions, the protein  
30 migrated as a Mr 50-55 kd polypeptide, as would be predicted if the mature product was glycosylated at one or more of its consensus N-linked glycosylation sites. Surprisingly, when the same samples were analysed by non-reducing SDS-PAGE, the majority of the protein  
35 migrated as an approximately 100 kd polypeptide, twice the size of the reduced protein. In addition, there

was a smaller amount of the Mr 50-55 kd polypeptide. This pattern of migration on SDS-PAGE was consistent with the notion that the OPG product was forming dimers through oxidation of a free sulfhydryl group(s).

5           The predicted mature OPG polypeptide contains 23 cysteine residues, 18 of which are predicted to be involved in forming intrachain disulfide bridges which comprise the four cysteine-rich domains (Figure 12A). The five remaining C-terminal cysteine residues are not  
10 involved in secondary structure which can be predicted based upon homology with other TNFR family members. Overall there is a net uneven number of cysteine residues, and it is formally possible that at least one  
15 bond between two OPG monomers.

          To help elucidate patterns of OPG kinesis and monomer association, a pulse-chase labelling study was performed. CHO cells expressing muOPG [22-401] were metabolically labelled as described above in serum-free  
20 medium containing <sup>35</sup>S methionine and cysteine for 30 min. After this period, the media was removed, and replaced with complete medium containing unlabelled methionine and cysteine at levels approximately 2,000-fold excess to the original concentration of  
25 radioactive amino acids. At 30 min, 1hr, 2 hr, 4 hr, 6 hr and 12 hr post addition, cultures were harvested by the removal of the conditioned media, and lysates of the conditioned media and adherent monolayers were prepared. The culture media and cell lysates were  
30 clarified as described above, and then immunoprecipitated using anti-OPG antibodies as described above. After the immunoprecipitates were washed, they were released by boiling in non-reducing SDS-PAGE buffer then split into two equal halves. To  
35 one half, the reducing agent  $\beta$ -mercaptoethanol was added to 5% (v/v) final concentration, while the other half

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was maintained in non-reducing conditions. Both sets of immunoprecipitates were analysed by SDS-PAGE as described above, then processed for autoradiography and exposed to film. The results are shown in Figure 16.

5 The samples analysed by reducing SDS-PAGE are depicted in the bottom two panels. After synthesis, the OPG polypeptide is rapidly processed to a slightly larger polypeptide, which probably represents modification by N-linked glycosylation. After approximately 1-2 hours,  
10 the level of OPG in the cell decreases dramatically, and concomitantly appears in the culture supernatant. This appears to be the result of the vectorial transport of OPG from the cell into the media over time, consistent with the notion that OPG is a naturally  
15 secreted protein. Analysis of the same immunoprecipitates under nonreducing conditions reveals the relationship between the formation of OPG dimers and secretion into the conditioned media (Figure 16, upper panels). In the first 30-60 minutes, OPG  
20 monomers are processed in the cell by apparent glycosylation, followed by dimer formation. Over time, the bulk of OPG monomers are driven into dimers, which subsequently disappear from the cell. Beginning about 60 minutes after synthesis, OPG dimers appear in  
25 the conditioned media, and accumulate over the duration of the experiment. Following this period, OPG dimers are formed, which are then secreted into the culture media. OPG monomers persist at a low level inside the cell over time, and small amounts also appear in the  
30 media. This does not appear to be the result of breakdown of covalent OPG dimers, but rather the production of sub-stoichiometric amounts of monomers in the cell and subsequent secretion.

Recombinantly produced OPG from transfected  
35 CHO cells appears to be predominantly a dimer. To determine if dimerization is a natural process in OPG

synthesis, we analysed the conditioned media of a cell  
line found to naturally express OPG. The CTLL-2 cell  
line, a murine cytotoxic T lymphocytic cell line (ATCC  
accession no. TIB-214), was found to express OPG mRNA  
5 in a screen of tissue and cell line RNA. The OPG  
transcript was found to be the same as the cloned and  
sequenced 2.5-3.0 kb RNA identified from kidney and  
found to encode a secreted molecule. Western blot  
analysis of conditioned media obtained from CTLL-2  
10 cells shows that most, if not all, of the OPG protein  
secreted is a dimer (Figure 17). This suggests that  
OPG dimerization and secretion is not an artifact of  
overexpression in a cell line, but is likely to be the  
main form of the product as it is produced by  
15 expressing cells.

Normal and transgenic mouse tissues and serum  
were analysed to determine the nature of the OPG  
molecule expressed in OPG transgenic mice. Since the  
rat OPG cDNA was expressed under the control of a  
20 hepatocyte control element, extracts made from the  
parenchyma of control and transgenic mice under non-  
reducing conditions were analysed (Figure 18). In  
extract from transgenic, but not control mice, OPG  
dimers are readily detected, along with  
25 substoichiometric amounts of monomers. The OPG dimers  
and monomers appear identical to the recombinant murine  
protein expressed in the genetically engineered CHO  
cells. This strongly suggests that OPG dimers are  
indeed a natural form of the gene product, and are  
30 likely to be key active components. Serum samples  
obtained from control and transgenic mice were  
similarly analysed by western blot analysis. In  
control mice, the majority of OPG protein migrates as a  
dimer, while small amounts of monomer are also  
35 detected. In addition, significant amounts of a larger  
OPG related protein is detected, which migrates with a

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relative molecular mass consistent with the predicted size of a covalently-linked trimer. Thus, recombinant OPG is expressed predominantly as a dimeric protein in OPG transgenic mice, and the dimer form may be the basis for the osteopetrotic phenotype in OPG mice. OPG recombinant protein may also exist in higher molecular weight "trimeric" forms.

To determine if the five C-terminal cysteine residues of OPG play a role in homodimerization, the murine OPG codons for cysteine residues 195 (C195), C202, C277, C319, and C400 were changed to serine using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) as described above. The muOPG gene was subcloned between the Not I and Xba I sites of the pcDNA 3.1 (+) vector (Invitrogen, San Diego, CA). The resulting plasmid, pcDNA3.1-muOPG, and mutagenic primers were treated with Pfu polymerase in the presence of deoxynucleotides, then amplified in a thermocycler as described above. An aliquot of the reaction is then transfected into competent E. coli XL1-Blue by heatshock, then plated. Plasmid DNA from transformants was then sequenced to verify mutations.

The following primer pairs were used to change the codon for cysteine residue 195 to serine of the murine OPG gene, resulting in the production of a muOPG [22-401] C195S protein:

1389-19:

5' -CAC GCA AAA GTC GGG AAT AGA TGT CAC-3'  
(SEQ ID NO:150)

1406-38:

5' -GTG ACA TCT ATT CCC GAC TTT TGC GTG-3'  
(SEQ ID NO:151)

5

10

(SEO ID NO:152)

15

(SEO ID NO:153)

20

(SEO ID NO:154)

25

(SEO ID NO:155)

30

35

(SEQ ID NO:156)

5' -CGA TTT CGA GGT CTT TCT CGT TCT C-3'  
(SEQ ID NO:157)

10 1406-72:

5' -CCG TGA AAA TAA GCT CGT TAT AAC TAG GAA TGG-3'  
(SEQ ID NO:158)

15            5' -CCA TTC CTA GTT ATA ACG AGC TTA TTT TCA CGG-3'  
(SEQ ID NO:159)

Each resulting muOPG [22-401] plasmid containing the appropriate mutation was then transfected into human 293 cells, the mutant OPG-Fc fusion protein purified from conditioned media as described above. The biological activity of each protein was assessed the in vitro osteoclast forming assay described in example 11. Conditioned media from each transfectant was analysed by non-reducing SDS-PAGE and western blotting with anti-OPG antibodies.

30 Mutation of any of the five C-terminal  
cysteine residues results in the production of  
predominantly (>90%) monomeric 55 kd OPG molecules.  
This strongly suggests that the C-terminal cysteine  
residues together play a role in OPG homodimerization.

C-terminal OPG deletion mutants were constructed to map the region(s) of the OPG C-terminal domain which are important for OPG homodimerization. These OPG mutants were constructed by PCR amplification using primers which introduce premature stop

translation signals in the C-terminal region of murine OPG. The 5' oligo was designed to the MuOPG start codon (containing a HindIII restriction site) and the 3' oligonucleotides (containing a stop codon and XhoI site) were designed to truncate the C-terminal region of muOPG ending at either threonine residue 200 (CT 200), proline 212 (CT212), glutamic acid 293 (CT-293), or serine 355 (CT-355).

The following primers were used to construct muOPG [22-200]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA  
ACA AG-3' (SEQ ID NO:160)

1391-91:

5' -CCT CTC TCG AGT CAG GTG ACA TCT ATT CCA CAC  
TTT TGC GTG GC-3' (1391-91) (SEQ ID NO:161)

The following primers were used to construct muOPG [22-212]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA  
ACA AG-3' (SEQ ID NO:162)

1391-90:

5' -CCT CTC TCG AGT CAA GGA ACA GCA AAC CTG AAG  
AAG GC -3' (SEQ ID NO:163)

The following primers were used to construct muOPG [22-293]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA  
ACA AG-3' (SEQ ID NO:164)

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1391-89:

5' - CCT CTC TCG AGT CAC TCT GTG GTG AGG TTC GAG  
TGG CC-3' (SEQ ID NO:165)

5 The following primers were used to construct muOPG  
[22-355]:

1091-39:

5' -CCT CTG AGC TCA AGC TTC CGA GGA CCA CAA TGA  
10 ACA AG-3' (SEQ ID NO:166)

1391-88:

5' CCT CTC TCG AGT CAG GAT GTT TTC AAG TGC TTG AGG GC-  
3'  
15 (SEQ ID NO:167)

Each resulting muOPG-CT plasmid containing  
the appropriate truncation was then transfected into  
human 293 cells, the mutant OPG-Fc fusion protein  
20 purified from conditioned media as described above.  
The biological activity of each protein was assessed  
the in vitro osteoclast forming assay described in  
example 11. The conditioned medias were also analysed  
by non-reducing SDS-PAGE and western blotting using  
25 anti-OPG antibodies.

Truncation of the C-terminal region of OPG  
effects the ability of OPG to form homodimers. CT 355  
is predominantly monomeric, although some dimer is  
formed. CT 293 forms what appears to be equal molar  
30 amounts of monomer and dimer, and also high molecular  
weight aggregates. However, CT 212 and CT 200 are  
monomeric.

EXAMPLE 10

Purification of OPG

A. Purification of mammalian OPG-Fc Fusion Proteins

5           5 L of conditioned media from 293 cells  
expressing an OPG-Fc fusion protein were prepared as  
follows. A frozen sample of cells was thawed into 10  
ml of 293S media (DMEM-high glucose, 1x L-glutamine,  
10% heat inactivated fetal bovine serum (FBS) and 100  
10 ug/ml hygromycin) and fed with fresh media after one  
day. After three days, cells were split into two T175  
flasks at 1:10 and 1:20 dilutions. Two additional 1:10  
splits were done to scale up to 200 T175 flasks. Cells  
were at 5 days post-thawing at this point. Cells were  
15 grown to near confluency (about three days) at which  
time serum-containing media was aspirated, cells were  
washed one time with 25 ml PBS per flask and 25 ml of  
SF media (DMEM-high glucose, 1x L-glutamine) was added  
to each flask. Cells were maintained at 5% CO<sub>2</sub> for  
20 three days at which point the media was harvested,  
centrifuged, and filtered through 0.45m cellulose  
nitrate filters (Corning).

OPG-Fc fusion proteins were purified using a  
Protein G Sepharose column (Pharmacia) equilibrated in  
25 PBS. The column size varied depending on volume of  
starting media. Conditioned media prepared as  
described above was loaded onto the column, the column  
washed with PBS, and pure protein eluted using 100mM  
glycine pH 2.7. Fractions were collected into tubes  
30 containing 1M Tris pH 9.2 in order to neutralize as  
quickly as possible. Protein containing fractions were  
pooled, concentrated in either an Amicon Centricon 10  
or Centriprep 10 and diafiltered into PBS. The pure  
protein is stored at -80°C.

35           Murine [22-401]-Fc, Murine [22-180]-Fc,  
Murine [22-194]-Fc, human [22-401]-Fc and human [22-



201]Fc were purified by this procedure. Murine [22-185]-Fc is purified by this procedure.

B. Preparation of anti-OPG antibodies

5           Three New Zealand White rabbits (5-8 lbs initial wt) were injected subcutaneously with muOPG[22-401]-Fc fusion protein. Each rabbit was immunized on day 1 with 50 µg of antigen emulsified in an equal volume of Freund's complete adjuvant. Further boosts  
10 (Days 14 and 28) were performed by the same procedure with the substitution of Freund's incomplete adjuvant. Antibody titers were monitored by EIA. After the second boost, the antisera revealed high antibody titers and 25ml production bleeds were obtained from each animal.  
15 The sera was first passed over an affinity column to which murine OPG-Fc had been immobilized. The anti-OPG antibodies were eluted with Pierce Gentle Elution Buffer containing 1% glacial acetic acid. The eluted protein was then dialyzed into PBS and passed over a Fc  
20 column to remove any antibodies specific for the Fc portion of the OPG fusion protein. The run through fractions containing anti-OPG specific antibodies were dialyzed into PBS.

25 C. Purification of murine OPG[22-401]

Antibody Affinity Chromatography

Affinity purified anti-OPG antibodies were diafiltered into coupling buffer (0.1M sodium carbonate  
30 pH 8.3, 0.5M NaCl), and mixed with CNBr-activated sepharose beads (Pharmacia) for two hours at room temperature. The resin was then washed with coupling buffer extensively before blocking unoccupied sites with 1M ethanolamine (pH 8.0) for two hours at room  
35 temperature. The resin was then washed with low pH (0.1M sodium acetate pH 4.0, 0.5M NaCl) followed by a

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high pH wash (0.1M Tris-HCl pH 8.0, 0.5M NaCl). The last washes were repeated three times. The resin was finally equilibrated with PBS before packing into a column. Once packed, the resin was washed with PBS. A  
5 blank elution was performed with 0.1M glycine-HCl, pH 2.5), followed by re-equilibration with PBS.

Concentrated conditioned media from CHO cells expressing muOPG[22-410] was applied to the column at a low flow rate. The column was washed with PBS until UV  
10 absorbance measured at 280nm returned to baseline. The protein was eluted from the column first with 0.1M glycine-HCl (pH 2.5), re-equilibrated with PBS, and eluted with a second buffer (0.1M CAPS, pH 10.5), 1M NaCl). The two elution pools were diafiltered  
15 separately into PBS and sterile filtered before freezing at -20°C.

#### Conventional Chromatography

CHO cell conditioned media was concentrated  
20 23x in an Amicon spiral wound cartridge (S10Y10) and diafiltered into 20mM tris pH 8.0. The diafiltered media was then applied to a Q-sepharose HP (Pharmacia) column which had been equilibrated with 20mM tris pH 8.0. The column was then washed until absorbance at  
25 280nm reached baseline. Protein was eluted with a 20 column volume gradient of 0-300mM NaCl in tris pH 8.0. OPG protein was detected using a western blot of column fractions.

Fractions containing OPG were pooled and  
30 brought to a final concentration of 300mM NaCl, 0.2mM DTT. A NiNTA superose (Qiagen) column was equilibrated with 20mM tris pH 8.0, 300mM NaCl, 0.2mM DTT after which the pooled fractions were applied. The column was washed with equilibration buffer until baseline  
35 absorbance was reached. Proteins were eluted from the column with a 0-30mM Imidazole gradient in

equilibration buffer. Remaining proteins were washed off the column with 1M Imidazole. Again a western blot was used to detect OPG containing fractions.

Pooled fractions from the NiNTA column were  
5 dialyzed into 10mM potassium phosphate pH 7.0, 0.2mM DTT. The dialyzed pool was then applied to a ceramic hydroxyapatite column (Bio-Rad) which had been equilibrated in 10mM phosphate buffer. After column washing, the protein was eluted with a 10-100mM  
10 potassium phosphate gradient over 20 column volumes. This was then followed by a 20 column volume gradient of 100-400 mM phosphate.

OPG was detected by coomassie blue staining of SDS-polyacrylamide gels and by western blotting.  
15 Fractions were pooled and diafiltered onto PBS and frozen at -80°C. The purified protein runs as a monomer and will remain so after diafiltration into PBS. The monomer is stable when stored frozen or at pH 5 at 4°C. However if stored at 4°C in PBS, dimers and what  
20 appears to be trimers and tetramers will form after one week.

D. Purification of human OPG met[22-401] from E. coli

25 The bacterial cell paste was suspended into 10 mM EDTA to a concentration of 15% (w/v) using a low shear homogenizer at 5°C. The cells were then disrupted by two homogenizations at 15,000 psi each at 5°C. The resulting homogenate was centrifuged at 5,000  
30 x g for one hour at 5°C. The centrifugal pellet was washed by low shear homogenization into water at the original homogenization volume followed by centrifugation as before. The washed pellet was then solubilized to 15% (w/v) by a solution of (final  
35 concentration) 6 M guanidine HCl, 10 mM dithiothreitol, 10 mM TrisHCl, pH 8.5 at ambient temperature for 30

minutes. This solution was diluted 30-fold into 2M urea containing 50 mM CAPS, pH 10.5, 1 mM reduced glutathione and then stirred for 72 hours at 5°C. The OPG was purified from this solution at 25°C by first  
5 adjustment to pH 4.5 with acetic acid and then chromatography over a column of SP-HP Sepharose resin equilibrated with 25 mM sodium acetate, pH 4.5. The column elution was carried out with a linear sodium chloride gradient from 50 mM to 550 mM in the same  
10 buffer using 20 column volumes at a flow rate of 0.1 column volumes/minute. The peak fractions containing only the desired OPG form were pooled and stored at 5°C or buffer exchanged into phosphate buffered saline, concentrated by ultrafiltration, and then stored at  
15 5°C. This material was analyzed by reverse phase HPLC, SDS-PAGE, limulus amebocyte lysate assay for the presence of endotoxin, and N-terminal sequencing. In addition, techniques such as mass spectrometry, pH/temperature stability, fluorescence, circular  
20 dichroism, differential scanning calorimetry, and protease profiling assays may also be used to examine the folded nature of the protein.

25

#### EXAMPLE 11

##### Biological Activity of Recombinant OPG

Based on histology and histomorphometry, it appeared that hepatic overexpression of OPG in  
30 transgenic mice markedly decreased the numbers of osteoclasts leading to a marked increase in bone tissue (see Example 4). To gain further insight into potential mechanism(s) underlying this in vivo effect, various forms of recombinant OPG have been tested in an  
35 in vitro culture model of osteoclast formation (osteoclast forming assay). This culture system was

originally devised by Udagawa (Udagawa et al.  
Endocrinology 125, 1805-1813 (1989), Proc. Natl. Acad.  
Sci. USA 87, 7260-7264 (1990)) and employs a  
combination of bone marrow cells and cells from bone  
5 marrow stromal cell lines. A description of the  
modification of this culture system used for these  
studies has been previously published (Lacey et al.  
Endocrinology 136, 2367-2376 (1995)). In this method,  
bone marrow cells, flushed from the femurs and tibiae  
10 of mice, are cultured overnight in culture media (alpha  
MEM with 10% heat inactivated fetal bovine serum)  
supplemented with 500 U/ml CSF-1 (colony stimulating  
factor 1, also called M-CSF), a hematopoietic growth  
factor specific for cells of the monocyte/macrophage  
15 family lineage. Following this incubation, the non-  
adherent cells are collected, subjected to gradient  
purification, and then cocultured with cells from the  
bone marrow cell line ST2 ( $1 \times 10^6$  non-adherent cells :  
 $1 \times 10^5$  ST2 cells/ ml media). The media is  
20 supplemented with dexamethasone (100 nM) and the  
biologically-active metabolite of vitamin D3 known as  
1,25 dihydroxyvitamin D3 (1,25 (OH)<sub>2</sub> D3, 10 nM). To  
enhance osteoclast appearance, prostaglandin E2 (250  
nM) is added to some cultures. The coculture period  
25 usually ranges from 8 - 10 days and the media, with all  
of the supplements freshly added, is renewed every 3-4  
days. At various intervals, the cultures are assessed  
for the presence of tartrate acid phosphatase (TRAP)  
using either a histochemical stain (Sigma Kit # 387A,  
30 Sigma, St. Louis, MO) or TRAP solution assay. The TRAP  
histochemical method allows for the identification of  
osteoclasts phenotypically which are multinucleated (  
3 nuclei) cells that are also TRAP+. The solution  
assay involves lysing the osteoclast-containing  
35 cultures in a citrate buffer (100 mM, pH 5.0)  
containing 0.1% Triton X-100. Tartrate resistant acid

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phosphatase activity is then measured based on the conversion of p-nitrophenylphosphate (20 nM) to p-nitrophenol in the presence of 80 mM sodium tartrate which occurs during a 3-5 minute incubation at RT. The  
5 reaction is terminated by the addition of NaOH to a final concentration of 0.5 M. The optical density at 405 nm is measured and the results are plotted.

Previous studies (Udagawa et al. ibid) using the osteoclast forming assay have demonstrated that  
10 these cells express receptors for  $^{125}\text{I}$ -calcitonin (autoradiography) and can make pits on bone surfaces, which when combined with TRAP positivity confirm that the multinucleated cells have an osteoclast phenotype. Additional evidence in support of the osteoclast  
15 phenotype of the multinucleated cells that arise in vitro in the osteoclast forming assay are that the cells express  $\alpha_v$  and  $\beta_3$  integrins by immunocytochemistry and calcitonin receptor and TRAP mRNA by in situ hybridization (ISH).

20 The huOPG [22-401]-Fc fusion was purified from CHO cell conditioned media and subsequently utilized in the osteoclast forming assay. At 100 ng/ml of huOPG [22-401]-Fc, osteoclast formation was virtually 100% inhibited (Figure 19A). The levels of  
25 TRAP measured in lysed cultures in microtitre plate wells were also inhibited in the presence of OPG with an  $\text{ID}_{50}$  of approximately 3 ng/ml (Figure 20). The level of TRAP activity in lysates appeared to correlate with the relative number of osteoclasts seen by TRAP  
30 cytochemistry (compare Figures 19A-19G and 20). Purified human IgG1 and TNFbp were also tested in this model and were found to have no inhibitory or stimulatory effects suggesting that the inhibitory effects of the huOPG [22-401]-Fc were due to the OPG  
35 portion of the fusion protein. Additional forms of the

human and murine molecules have been tested and the cumulative data are summarized in Table 1.

5

Table 1  
Effects of various OPG forms on in vitro  
osteoclast formation

10	<u>OPG Construct</u>	<u>Relative Bioactivity in vitro</u>
	muOPG [22-401]-Fc	+++
	muOPG [22-194]-Fc	+++
	muOPG [22-185]-Fc	++
15	muOPG [22-180]-Fc	-
	muOPG [22-401]	+++
	muOPG [22-401] C195	+++
	muOPG [22-401] C202	+
	muOPG [22-401] C277	-
20	muOPG [22-401] C319	+
	muOPG [22-401] C400	+
	muOPG [22-185]	-
	muOPG [22-194]	++
	muOPG [22-200]	++
25	muOPG [22-212]	-
	muOPG [22-293]	+++
	muOPG [22-355]	+++
	huOPG [22-401]-Fc	+++
30	huOPG [22-201]-Fc	+++
	huOPG [22-401]-Fc P26A	+++
	huOPG [22-401]-Fc Y28F	+++
	huOPG [22-401]	+++
	huOPG [27-401]-Fc	++
35	huOPG [29-401]-Fc	++
	huOPG [32-401]-Fc	+/-

- +++ ,  $ED_{50} = 0.4-2$  ng/ml  
++ ,  $ED_{50} = 2-10$  ng/ml  
+ ,  $ED_{50} = 10-100$  ng/ml  
5 - ,  $ED_{50} > 100$  ng/ml

The cumulative data suggest that murine and human OPG amino acid sequences 22-401 are fully active in vitro, when either fused to the Fc domain, or  
10 unfused. They inhibit in a dose-dependent manner and possess half-maximal activities in the 2-10 ng/ml range. Truncation of the murine C-terminus at threonine residue 180 inactivates the molecule, whereas truncations at cysteine 185 and beyond have full  
15 activity. The cysteine residue located at position 185 is predicted to form an SS3 bond in the domain 4 region of OPG. Removal of this residue in other TNFR-related proteins has previously been shown to abrogate biological activity (Yan et al. J. Biol. Chem. 266,  
20 12099-12104 (1994)). Our finding that muOPG[22-180]-Fc is inactive while muOPG[22-185]-Fc is active is consistent with these findings. This suggests that amino acid residues 22-185 define a region for OPG activity.

25 These findings indicate that like transgenically-expressed OPG, recombinant OPG protein also suppressed osteoclast formation as tested in the osteoclast forming assay. Time course experiments examining the appearance of TRAP+ cells,  $\beta 3$ + cells,  
30 F480+ cells in cultures continuously exposed to OPG demonstrate that OPG blocks the appearance TRAP+ and  $\beta 3$ + cells, but not F480+ cells. In contrast, TRAP+ and  $\beta 3$ + cells begin to appear as early as day 4 following culture establishment in control cultures. Only F480+  
35 cells can be found in OPG-treated cultures and they



appear to be present at qualitatively the same numbers as the control cultures. Thus, the mechanism of OPG effects in vitro appears to involve a blockade in osteoclast differentiation at a step beyond the  
5 appearance of monocyte-macrophages but before the appearance of cells expressing either TRAP or  $\beta 3$  integrins. Collectively these findings indicate that OPG does not interfere with the general growth and differentiation of monocyte-macrophage precursors from  
10 bone marrow, but rather suggests that OPG specifically blocks the selective differentiation of osteoclasts from monocyte-macrophage precursors.

To determine more specifically when in the osteoclast differentiation pathway that OPG was  
15 inhibitory, a variation of the in vitro culture method was employed. This variation, described in (Lacey et al. supra), employs bone marrow macrophages as osteoclast precursors. The osteoclast precursors are derived by taking the nonadherent bone marrow cells  
20 after an overnight incubation in CSF-1/M-CSF, and culturing the cells for an additional 4 days with 1,000 - 2,000 U/ml CSF-1. Following 4 days of culture, termed the growth phase, the non-adherent cells are removed. The adherent cells, which are bone marrow  
25 macrophages, can then be exposed for up to 2 days to various treatments in the presence of 1,000 - 2,000 U/ml CSF-1. This 2 day period is called the intermediate differentiation period. Thereafter, the cell layers are again rinsed and then ST-2 cells ( $1 \times 10^5$  cell/ml), dexamethasone (100 nM) and 1,25 (OH) $_2$  D $_3$  (10 nM) are added for the last 8 days for what is  
30 termed the terminal differentiation period. Test agents can be added during this terminal period as well. Acquisition of phenotypic markers of osteoclast  
35 differentiation are acquired during this terminal period (Lacey et al. ibid).

huOPG [22-401]-Fc (100 ng/ml) was tested for its effects on osteoclast formation in this model by adding it during either the intermediate, terminal or, alternatively, both differentiation periods. Both TRAP  
5 cytochemistry and solution assays were performed. The results of the solution assay are shown in Figure 21. HuOPG [22-401]-Fc inhibited the appearance of TRAP activity when added to both the intermediate and terminal or only the terminal differentiation phases.  
10 When added to the intermediate phase and then removed from the cultures by rinsing, huOPG [22-401]-Fc did not block the appearance of TRAP activity in culture lysates. The cytochemistry results parallel the solution assay data. Collectively, these observations  
15 indicate that huOPG [22-401]-Fc only needs to be present during the terminal differentiation period for it to exert its all of its suppressive effects on osteoclast formation.

B. In vivo IL1- $\alpha$  and IL1- $\beta$  challenge experiments

20 IL1 increases bone resorption both systemically and locally when injected subcutaneously over the calvaria of mice (Boyce et al., Endocrinology 125, 1142-1150 (1989)). The systemic effects can be assessed by the degree of hypercalcemia and the local  
25 effects histologically by assessing the relative magnitude of the osteoclast-mediated response. The aim of these experiments was to determine if recombinant muOPG [22-401]-Fc could modify the local and/or systemic actions of IL1 when injected subcutaneously  
30 over the same region of the calvaria as IL1.

IL-1  $\beta$  experiment

Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per  
35 group): Control group: IL1 treated animals (mice received 1 injection/day of 2.5 ug of IL1- $\beta$ ); Low dose

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muOPG [22-401]-Fc treated animals (mice received 3 injections/day of 1  $\mu$ g of muOPG [22-401]-Fc); Low dose muopg [22-401]-Fc and IL1- $\beta$ ; High dose muOPG [22-401]-Fc treated animals (mice receive 3 injections/day of 10  $\mu$ g muOPG [22-401]-Fc); High dose muOPG [22-401]-Fc and IL1- $\beta$ . All mice received the same total number of injections of either active factor or vehicle (0.1% bovine serum albumin in phosphate buffered saline). All groups are sacrificed on the day after the last injection. The weights and blood ionized calcium levels are measured before the first injections, four hours after the second injection and 24 hours after the third IL1 injection, just before the animals were sacrificed. After sacrifice the calvaria were removed and processed for paraffin sectioning.

#### IL1- $\alpha$ experiment

Male mice (ICR Swiss white) aged 4 weeks were divided into the following treatment groups (5 mice per group): Control group; IL1 alpha treated animals (mice received 1 injection/day of 5 ug of IL1-alpha); Low dose muOPG [22-401]-Fc treated animals (mice received 1 injection/day of 10  $\mu$ g of muOPG [22-401]-Fc; Low dose muopg [22-401]-Fc and IL1-alpha, (dosing as above); High dose muopg [22-401]-Fc treated animals (mice received 3 injections/day of 10  $\mu$ g muOPG [22-401]-Fc; High dose muOPG [22-401]-Fc and IL1- $\alpha$ . All mice received the same number of injections/day of either active factor or vehicle. All groups were sacrificed on the day after the last injection. The blood ionized calcium levels were measured before the first injection, four hours after the second injection and 24 hours after the third IL1 injection, just before the animals were sacrificed. The animal weights were measured before the first injection, four hours after the second injection and 24 hours after the third IL1

injection, just before the animals were sacrificed. After sacrifice the calvaria were removed and processed for paraffin sectioning.

5

#### Histological methods

Calvarial bone samples were fixed in zinc formalin, decalcified in formic acid, dehydrated through ethanol and mounted in paraffin. Sections (5µm  
10 thick) were cut through the calvaria adjacent to the lambdoid suture and stained with either hematoxylin and eosin or reacted for tartrate resistant acid phosphatase activity (Sigma Kit# 387A) and counterstained with hematoxylin. Bone resorption was  
15 assessed in the IL1-α treated mice by histomorphometric methods using the Osteomeasure (Osteometrics, Atlanta, GA) by tracing histologic features onto a digitizer platen using a microscope-mounted camera lucida attachment. Osteoclast numbers, osteoclast lined  
20 surfaces, and eroded surfaces were determined in the marrow spaces of the calvarial bone. The injected and non-injected sides of the calvaria were measured separately.

#### 25 Results

IL1-α and IL1-β produced hypercalcemia at the doses used, particularly on the second day, presumably by the induction of increased bone resorption systemically. The hypercalcemic response was blocked  
30 by muOPG [22-401]-Fc in the IL1-beta treated mice and significantly diminished in mice treated with IL1-alpha, an effect most apparent on day 2 (Figure 22A-22B).

Histologic analysis of the calvariae of mice  
35 treated with IL1-alpha and beta shows that IL1 treatments alone produce a marked increase in the

The most likely explanation for these observations is that muOPG [22-401]-Fc inhibited bone resorption, a conclusion supported by the reduction of both the total osteoclast number and the percentage of available bone surface undergoing bone resorption, in the region of the calvaria adjacent to the muOPG [22-401]-Fc injection sites. The actions of muOPG [22-401]-Fc appeared to be most marked locally by histology, but the fact that muOPG [22-401]-Fc also blunted IL1-induced hypercalcemia suggests that muOPG [22-401]-Fc has more subtle effects on bone resorption systemically.

Table 2. Effects of OPG on variables of bone resorption in IL-1 injected mice.

[illegible]

C. Systemic Effects of muOPG [22-401]-Fc in Growing Mice

Male BDF1 mice aged 3-4 weeks, weight range 9.2- 15.7g were divided into groups of ten mice per group. These mice were injected subcutaneously with saline or muOPG [22-401]-Fc 2.5mg/kg *bid* for 14 days (5mg/kg/day). The mice were radiographed before treatment, at day 7 and on day 14. The mice were sacrificed 24 hours after the final injection. The right femur was removed, fixed in zinc formalin, decalcified in formic acid and embedded in paraffin. Sections were cut through the mid region of the distal femoral metaphysis and the femoral shaft. Bone density, by histomorphometry, was determined in six adjacent regions extending from the metaphyseal limit of the growth plate, through the primary and secondary spongiosa and into the femoral diaphysis (shaft). Each region was 0.5 X 0.5 mm<sup>2</sup>.

Radiographic changes

After seven days of treatment there was evidence of a zone of increased bone density in the spongiosa associated with the growth plates in the OPG treated mice relative to that seen in the controls. The effects were particularly striking in the distal femoral and the proximal tibial metaphases (Figure 24A-24B). However bands of increased density were also apparent in the vertebral bodies, the iliac crest and the distal tibia. At 14 days, the regions of opacity had extended further into the femoral and tibial shafts though the intensity of the radio-opacity was diminished. Additionally, there were no differences in the length of the femurs at the completion of the experiment or in the change in length over the duration

of the experiment implying that OPG does not alter bone growth.

#### Histological Changes

5           The distal femoral metaphysis showed  
increased bone density in a regions 1.1 to 2.65 mm in  
distance from the growth plate (Figures 25 and 26A-  
26B). This is a region where bone is rapidly removed  
by osteoclast-mediated bone resorption in mice. In  
10 these rapidly growing young mice, the increase in bone  
in this region observed with OPG treatment is  
consistent with an inhibition of bone resorption.

#### D. Effects of Osteoprotegerin on Bone Loss Induced by 15 Ovariectomy in the Rat

Twelve week old female Fisher rats were  
ovariectomized (OVX) or sham operated and dual xray  
absorptiometry (DEXA) measurements made of the bone  
20 density in the distal femoral metaphysis. After 3  
days recovery period, the animals received daily  
injections for 14 days as follows: Ten sham operated  
animals received vehicle (phosphate buffered saline);  
Ten OVX animals received vehicle (phosphate buffered  
25 saline); Six OVX animals received OPG-Fc 5mg/kg SC;  
Six OVX animals received pamidronate (PAM) 5mg/kg SC;  
Six OVX animals received estrogen (ESTR) 40ug/kg SC.  
After 7 and 14 days treatment the animals had bone  
density measured by DEXA. Two days after the last  
30 injection the animals were killed and the right tibia  
and femur removed for histological evaluation.

The DEXA measurements of bone density showed a  
trend to reduction in the bone density following  
35 ovariectomy that was blocked by OPG-Fc. Its effects  
were similar to the known antiresorptive agents  
estrogen and pamidronate. (Figure 27). The



histomorphometric analysis confirmed these observations with OPG-Fc treatment producing a bone density that was significantly higher in OVX rats than that seen in untreated OVX rats (Figure 28). These results confirm the activity of OPG in the bone loss associated with withdrawal of endogenous estrogen following ovariectomy.

10 In vivo Summary

The in vivo actions of recombinant OPG parallel the changes seen in OPG transgenic mice. The reduction in osteoclast number seen in the OPG transgenic is reproduced by injecting recombinant OPG locally over the calvaria in both normal mice and in mice treated with IL1- $\alpha$  or IL1- $\beta$ . The OPG transgenic mice develop an osteopetrotic phenotype with progressive filling of the marrow cavity with bone and unremodelled cartilage extending from the growth plates from day 1 onward after birth. In normal three week old (growing) mice, OPG treatments also led to retention of bone and unremodelled cartilage in regions of endochondral bone formation, an effect observed radiographically and confirmed histologically. Thus, recombinant OPG produces phenotypic changes in normal animals similar to those seen in the transgenic animals and the changes are consistent with OPG-induced inhibition of bone resorption. Based on in vitro assays of osteoclast formation, a significant portion of this inhibition is due to impaired osteoclast formation. Consistent with this hypothesis, OPG blocks ovariectomy-induced osteoporosis in rat. Bone loss in this model is known to be mediated by activated osteoclasts, suggesting a role for OPG in treatment of primary osteoporosis.

## EXAMPLE 12

### Pegylation Derivatives of OPG

5

#### Preparation of N-terminal PEG-OPG conjugates by reductive alkylation

10 HuOPG met [22-194] P25A was buffer exchanged into  
25-50 mM NaOAc, pH 4.5-4.8 and concentrated to 2-5  
mg/ml. This solution was used to conduct OPG reductive  
alkylation with monofunctional PEG aldehydes at 5-7 °C.  
PEG monofunctional aldehydes, linear or branched, MW=1  
to 57 kDa (available from Shearwater Polymers) were  
added to the OPG solution as solids in amounts  
15 constituting 2-4 moles of PEG aldehyde per mole of OPG.  
After dissolution of polymer into the protein solution,  
sodium cyanoborohydride was added to give a final  
concentration of 15 to 20 mM in the reaction mixture  
from 1-1.6 M freshly prepared stock solution in cold DI  
20 water. The progress of the reaction and the extent of  
OPG PEGylation was monitored by size exclusion HPLC on  
a G3000SWXL column (Toso Haas) eluting with 100 mM  
NaPO<sub>4</sub>, 0.5 M NaCl, 10% ethanol, pH 6.9. Typically the  
reaction was allowed to proceed for 16-18 hours, after  
25 which the reaction mixture was diluted 6-8 times and  
the pH lowered to 3.5-4. The reaction mixture was  
fractionated by ion exchange chromatography (HP SP  
HiLoad 16/10, Pharmacia) eluting with 20 mM NaOAc pH 4  
with a linear gradient to 0.75M NaCl over 25 column  
30 volumes at a flow rate of 30 cm/h. Fractions of mono-,  
di- or poly- PEGylated OPG were pooled and  
characterized by SEC HPLC and SDS-PAGE. By N-terminal  
sequencing, it was determined that the monoPEG-OPG  
conjugate, the major reaction product in most cases,  
35 was 98% N-terminally PEG-modified OPG.

This procedure was generally used to prepare the following N-terminal PEG-OPG conjugates (where OPG is HuOPG met [22-194] P25A: 5 kD monoPEG, 10 kD mono branched PEG, 12 kD monoPEG, 20 kD monoPEG, 20 kD mono branched PEG, 25 kD monoPEG, 31 kD monoPEG, 57 kD monoPEG, 12 kD diPEG, 25 kD diPEG, 31 kD diPEG, 57 kD diPEG, 25 kD triPEG.

Preparation of PEG-OPG conjugates by acylation

10 HuOPG met [22-194] P25A was buffer exchanged into 50 mM BICINE buffer, pH 8 and concentrated to 2-3 mg/ml. This solution was used to conduct OPG acylation with monofunctional PEG N-hydroxysuccinimidyl esters at room temperature. PEG N-hydroxysuccinimidyl esters, 15 linear or branched, MW=1 to 57 kDa (available from Shearwater Polymers) were added to the OPG solution as solids in amounts constituting 4-8 moles of PEG N-hydroxysuccinimidyl ester per mole of OPG. The progress of the reaction and the extent of OPG PEGylation was 20 monitored by size exclusion HPLC on a G3000SWXL column (Toso Haas) eluting with 100 mM NaPO<sub>4</sub>, 0.5 M NaCl, 10% ethanol, pH 6.9. Typically the reaction was allowed to proceed for 1 hour, after which the reaction mixture was diluted 6-8 times and the pH lowered to 3.5-4. The 25 reaction mixture was fractionated by ion exchange chromatography (HP SP HiLoad 16/10, Pharmacia) eluting with 20 mM NaOAc pH 4 with a linear gradient to 0.75M NaCl over 25 column volumes at a flow rate of 30 cm/h. Fractions of mono-, di- or poly- PEGylated OPG were 30 pooled and characterized by SEC HPLC and SDS-PAGE.

This procedure was generally used to prepare the following PEG-OPG conjugates: 5 kD polyPEG, 20 kD polyPEG, 40 kD poly branched PEG, 50 kD poly PEG.

Preparation of dimeric PEG-OPG

HuOPG met [22-194] P25A is prepared for thiolation at 1-3 mg/ml in a phosphate buffer at near neutral pH. S-acetyl mecaptosuccinic anhydride (AMSA) is added in a 3-7 fold molar excess while maintaining pH at 7.0 and the rxn stirred at 4°C for 2 hrs. The monothiolated-OPG is separated from unmodified and polythiolated OPG by ion exchange chromatography and the protected thiol deprotected by treatment with hydroxylamine. After deprotection, the hydroxylamine is removed by gel filtration and the resultant monothiolated-OPG is subjected to a variety of thiol specific crosslinking chemistries. To generate a disulfide bonded dimer, the thiolated OPG at >1mg/ml is allowed to undergo air oxidation by dialysis in slightly basic phosphate buffer. The covalent thioether OPG dimer was prepared by reacting the bis-maleimide crosslinker, N,N-bis(3-maleimido propianyl)-2-hydroxy 1,3 propane with the thiolated OPG at >1mg/ml at a 0.6x molar ratio of crosslinker:OPG in phosphate buffer at pH 6.5. Similarly, the PEG dumbbells are produced by reaction of substoichiometric amounts of bis-maleimide PEG crosslinkers with thiolated OPG at >1mg/ml in phosphate buffer at pH 6.5. Any of the above dimeric conjugates may be further purified using either ion exchange or size exclusion chromatographies.

Dimeric PEG-OPG conjugates (where OPG is HuOPG met [22-194] P25A prepared using the above procedures include disulfide-bonded OPG dimer, covalent thioether OPG dimer with an aliphatic amine type crosslinker, 3.4 kD and 8kD PEG dumbbells and monobells.

PEG-OPG conjugates were tested for activity in vitro using the osteoclast maturation assay described in Example 11A and for activity in vivo by measuring increased bone density after injection into mice as

described in Example 11C. The in vivo activity is shown below in Table 3.

Table 3

5 In vivo biological activity of Pegylated OPG

<u>OPG Construct</u>		<u>Increase in Tibial Bone Density</u>
	muOPG met [22-194]	-
10	muOPG met [22-194] 5k PEG	+
	muOPG met [22-194] 20k PEG	+
	huOPG met [22-194] P25A	-
	huOPG met [22-194] P25A 5k PEG	+
15	huOPG met [22-194] P25A 20k PEG	+
	huOPG met [22-194] P25A 31k PEG	+
	huOPG met [22-194] P25A 57k PEG	+
	huOPG met [22-194] P25A 12k PEG	+
	huOPG met [22-194] P25A 20k Branched PEG	+
20	huOPG met [22-194] P25A 8k PEG dimer	+
	huOPG met [22-194] P25A disulfide crosslink	+

\* \* \*

25 While the invention has been described in what is considered to be its preferred embodiments, it is not to be limited to the disclosed embodiments, but on the contrary, is intended to cover various modifications and equivalents included within the  
30 spirit and scope of the appended claims, which scope is to be accorded the broadest interpretation so as to encompass all such modifications and equivalents.